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THE EFFECTS OF FOUR TETRACYCLINE DRUGS ON ANIMAL GROWTH AND HARD TISSUE DEVELOPMENT IN RATS

by

DAMAN D. THANIK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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OF MASTER OF SCIENCE

IN

ORAL BIOLOGY

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommended to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Effects of Four Tetracycline Drugs on Animal Growth and Hard Tissue Development in Rats." Submitted by Daman D. Thanik in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

It is usually assumed that tetracyclines giving minimal discoloration in bone are likely to be less toxic. In order to investigate this point the effects of tetracycline, oxytetracycline, chlortetracycline and demethylchlortetracycline in doses ranging from 1/4 to 5 times the normal (40 mg/kg) have been studied in terms of general body growth, length, volume and density of femur, and color intensity exhibited by bone and teeth under u.v. light. Male rats were injected I.P. once daily for 6 days and then sacrificed after a rest period of about 2 weeks. The growth curves of the animals indicated that though OTC and CTC were toxic at 200 mg/kg and caused weight loss and death of the animals respectively, CTC caused cessation of growth even at 80 mg/kg. At comparable dosage DCTC has little effect on the growth of the animal. The color intensities exhibited in the bones and teeth were directly dependent upon the doses administered. OTC gave the least discoloration (creamy yellowish) whereas DCTC gave the most intense (yellowish orange) at comparable levels of drug administration. It is interesting to note that while OTC gives the least discoloration it is observed to be one of the drugs most inhibiting to growth at high dosage. Also OTC causes a comparatively greater decrease in femur length, volume and density. In comparison TC and DCTC gave intense discoloration but no appreciable changes occurred in femur length and volume, although a significant decrease is observed in bone density. Thus, it is suggested that the toxicity of the tetracyclines cannot be based on color intensity observations only.

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INTRODUCTION

Many investigations have been made of tetracycline drugs in the last 17 years with a view to gaining a better insight into their beneficial and undesirable effects both in animals and humans. In recent years it has been reported that these drugs become incorporated in tissues undergoing mineralization at the time the drugs are administered. These drugs are commonly used to combat infections in the mother during pregnancy and in the child early in life. Since large numbers of deciduous teeth start their morphodifferentiation in the foetal life and their growth and development continue until the end of first year of age, so it is quite conceivable that any systemic intake of the drugs would influence the teeth and bones directly by one or both of the following pathways: (1) by affecting the teeth and bones during the period of morphodifferentiation and development and (2) by affecting the teeth and bones during the process of mineralization. If the skeletal tissues are affected during the formative stage, then inhibition of growth, reduction in skeletal size and hypoplasia in case of teeth should result, whereas the drugs given during the mineralization phase would either depress mineralization or get incorporated in the calcifying tissues with other mineral salts. There are published reports to the fact that these drugs cause inhibition in growth and leave discoloration and fluorescent colored markings in the bone and teeth. Over a period of time the fluorescence is lost completely from the bone as it is being continuously resorbed and remodelled, but the discoloration and fluorescence left by these drugs in calcifying tissues like enamel and dentine is permanent and persist throughout the life of the individual.

Since their use is essential in controlling serious infections of pregnancy and early childhood diseases such as cystic fibrosis of the pancreas, these beneficial effects may outweigh the drawbacks of tooth discoloration, but it is felt, that later could be minimized by selection of the least offensive of the four tetracyclines and by avoiding their use during the period of development of the crown portions of the anterior deciduous and permanent teeth. Thus it was thought to be of value to investigate the following:

(1) Comparison of four commonly used tetracycline drugs at four dose levels in respect to their effect on animal and bone growth.

(2) What is the effect of these drugs on the mineralization of bone and teeth?

(3) Are the fluorescent markings hypomineralized?

(4) Comparison of the fluorescent color intensity given by these drugs and whether or not the color intensity is dependent upon the dose level administered.

In laying a foundation for our investigation, the literature associated directly, and sometimes indirectly, to this problem has been reviewed. In doing this, reports of experiments in respect to bone and dental tissues have been separated even though this detracts somewhat from their significance. Finally, the main purpose of this type of investigation is that of a teaching exercise, and to familiarize the student in the many and varied steps necessary to execute the previously thought of experimental design.

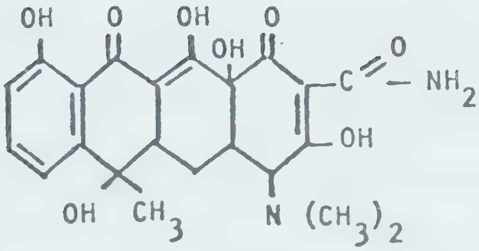
REVIEW OF LITERATURE

I. TETRACYCLINES AS DRUGS

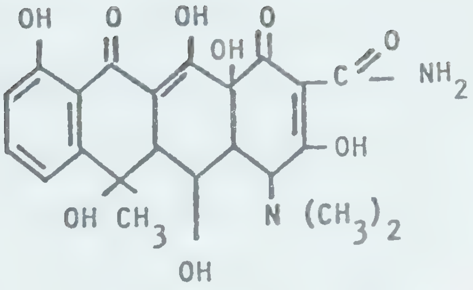
Extensive surveys to discover new antibiotics from soil actinomycetes led to the discovery of streptomycin and chloromycetin (presently known as chloramphenicol). In 1948 Duggar[1] isolated a pale yellow substance from the then unknown species of Streptomyces aureofaciens, and it was given the name Aureomycin. About two years later (1950) another antibiotic was isolated from a new species called Streptomyces remusus and it was given the name Terramycin[2]. As research progressed Stephens _et al_[3] reported that both these drugs appear to have similar antibacterial spectrums and are also chemically related. As a result of their chemical configuration the two antibiotics were renamed chlortetracycline (Aureomycin) and oxytetracycline (Terramycin).

Boothe _et al_[4] (1953) and Conover _et al_[5] (1953) reported another new antibacterial compound called tetracycline prepared by catalytic dehalogenation of chlortetracycline. This antibiotic was prepared independently in the laboratories of Lederle and Pfizer. In 1957 McCormick _et al_[6] and others reported the discovery of a fourth antibiotic DCTC which was produced by the mutant strain of Streptomyces aureofaciens. The rapid scientific progress in this field finally led in 1962 to the complete synthesis of tetracycline type antibiotics.

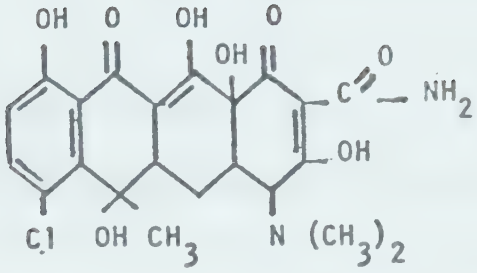
The molecular structure of the above mentioned four tetracyclines is basically the same. They differ from each other in respect to the location of the Cl, OH and CH_3 groups in the configuration of the tetracycline molecule.



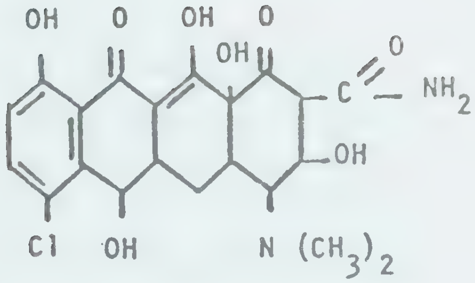
TETRACYCLINE



OXYTETRACYCLINE



CHLORTETRACYCLINE



DEMETHYLCHLORTETRACYCLINE

Of the four drugs, chlortetracycline is the most unstable *in vitro*. It is photosensitive both in solution and powder forms. Solutions of CTC are unstable at room temperature. They remain perfectly stable for 4-7 days at 4°C. All the drugs are quite stable at an acid pH but lose much of their activity at neutral or higher pH levels. Bases of these drugs are very slightly soluble, but in the form of their salts they are readily soluble in distilled water and solubility further increases

as the pH is lowered.

In general, antimicrobial activity of these drugs is similar but not identical. Many authors[7-11] have studied the comparative activity of these drugs and have reported almost similar results. The studies have shown that CTC and DCTC were most active against most species of gram-positive bacteria, DCTC giving slightly better results than CTC. TC was usually the most active against gram-negative bacilli. Except for some strains, OTC gave the worst over-all result in comparison to other drugs.

In a review by Finland and Garrod[12], DCTC is mentioned to be more active than tetracycline by a factor of 2. On a weight basis DCTC is the most active, and thus has the advantage that it may be given in smaller doses and at less frequent intervals.

Though tetracycline drugs can be prepared in suitable forms for intravenous, intramuscular and intraperitoneal injections the usual mode of administration is oral, in capsules containing the hydrochloride. Intramuscular injections are very painful owing to acid pH of the solutions. Suitably buffered preparations in Procaine hydrochloride base are employed for intramuscular injection.

Each of the four tetracyclines is well enough absorbed from the gastro-intestinal tract after oral administration for therapeutic effectiveness and are excreted in both the urine and faeces regardless of the route of administration[25,26]. After oral administration peak level usually occurs within 2-6 hours. The blood concentration level is proportionate to the dose in amounts up to 1 gm but further increases of the dose does not lead to significantly higher blood concentrations. Tetracycline gives the highest and best sustained blood levels and CTC

the lowest and least well maintained[13]. This is probably due to greater instability of chloretracycline. Studies[12,14,15] have shown that DCTC produces higher sustained blood levels than any of the other three tetracyclines and a measureable level of the drug can be detected even 48 hours after a single oral dose.

Absorption of tetracyclines from the alimentary canal is never complete, and the larger the dose the lower is the proportion of it absorbed. Two factors appear to be involved. Firstly, the hydrochlorides are reasonably soluble in water, giving highly acidic solutions, but in neutral or alkaline environment they tend to be precipitated. This situation could occur when liberated in the intestine. Secondly, the tetracyclines combine with divalent metals (e.g. calcium) in the alimentary canal, which reduces the available amount of the drug to be absorbed. Welch et al[16] and Sweeney et al[17] studied the effect of additions of salts to the contents of tetracycline capsules on the blood levels attained, and reported that calcium phosphate reduced absorption, whereas sodium metaphosphate or citric acid increased it substantially. Thus, in order to enhance the absorption, sodium phosphate is commonly included in the capsule either as an addition or in combination with antibiotic as a tetracycline phosphate complex[18]. The sodium phosphate combines with calcium in the intestinal tract and thus renders it unavailable for combination with the antibiotic.

Parenteral administration of the drugs yields concentrations as much as 4-6 times higher than achieved by oral route.

Tetracyclines behave much like penicillin in their diffusion into serous cavities, the foetal circulation and glandular secretions. All the tetracyclines deposit in the bone in areas where new bone is being

laid down.

Tetracyclines are freely excreted in bile, urine and faeces. Certain amounts are also present in the saliva. Concentrations excreted in bile are 10-20 times those in the blood; much of the antibiotic so excreted is reabsorbed[19,20]. Helander and Bottiger[21] and Bottiger[22,23] studied the absorption, distribution, storage and excretion of OTC, CTC and TC in experimental animals using fluorescence microscopy and showed that antibiotic passed from the intestine into the blood stream, then into the liver and then back into the intestine, thus establishing a circuit. Urinary excretion accounts for over 20 percent of an oral dose and 50 percent of an intravenous dose. The difference between these two is explained by incomplete absorption from the intestinal tract or possible degradation in the body. Often certain amounts are excreted in faeces when administered orally[25] or parenterally[22,23].

Kunin _et al_[24] made a comparative study of the renal clearance and plasma binding of the four tetracyclines. They found that only 18 percent of CTC was excreted in urine by 96 hours after intravenous injection in comparison to 70 percent of OTC, 60 percent of TC and 42 percent of DCTC. Lower figures obtained with CTC are explained because of a higher degree of plasma binding. Kelly and Buyske[25], Kelly, Kanegis and Buyske[26] and Eisner and Wulf[27] studied the metabolism of radioactive TC, DCTC and CTC in dogs and rats and reported that

> "90 percent of the administered radioactivity was eliminated by either the urinary or faecal route. A significant portion of the remaining activity was bound as a chelated tetracycline in the skeleton of the animal."

They further showed that tetracyclines are excreted in both the urine and faeces regardless of the route of administration of radioactive tetracyclines,

> "significant amounts of radioactivity were excreted via the faeces. CTC was excreted to the greatest extent (40 to 50% of the dose) by this pathway, followed in order by DCTC 15 to 30% and TC 9 to 20%."

None of these drugs was shown to have undergone any metabolic change in the bodies of rats or dogs.

A comparative distribution of the tetracyclines in animals has been studied quite extensively by many investigators[22,23,26,28-30]. Researches of Welch[28], Helander and Bottiger[21], Bottiger[22,23] and Eidus[30] have shown that tetracyclines were widely distributed in various organs and soft tissue. The highest concentrations were found in the reticuloendothelial system, and in liver, kidney, spleen and intestinal tract. After a period of 96 hours following I.V. administration no drug could be found in the organs and soft tissues except the skeletal system.

Tetracycline drugs have been reported to cause gastrointestinal disturbances. Commonly known symptoms are nausea, vomiting and diarrhea. Generally it is agreed that tetracycline is the least offender and OTC is the worst[7,8]. The causes of gastrointestinal disturbances are not entirely clear. The following factors may be responsible:

(1) Inhibition or complete disappearance of certain members of the normal bacterial flora which render the bowel susceptible to invasion by other microbes[8] or to overgrowth by those normally found in small numbers.

(2) Chemical irritation, due to acidic pH of the drugs especially after long continued oral administration.

(3) Inhibition of digestive enzymes, e.g., pancreatic enzymes, amylase and lipase, thus leading to a decrease in the absorption of glucose, starch and the fats[31].

It is quite clear from many studies[21-23] that these drugs accumulate in the soft tissues, namely liver, kidney, spleen, etc., and their continued presence there may cause damage. Prolonged therapy with tetracyclines has been shown to cause liver damage. Lepper _et al_[32] observed severe liver damage in patients treated with heavy doses of chlortetracycline. Later[33] they confirmed the above observations in experiments on mice and dogs.

Schults and associates[34] reported the deaths of 6 pregnant women who had been receiving large doses of tetracyclines intravenously. Since this report, many similar instances[35-39] have been reported in which young pregnant women receiving high doses of tetracyclines for complications of pregnancy developed hepatotoxicity. Whalley _et al_[37] believe that pregnancy and possibly infections may provide conditions for tetracycline toxicity to be manifested. The main findings revealed at the time of autopsy are usually diffuse fine droplet fatty metamorphosis of the liver. According to Dowling and Lepper[38], the factors which appear to contribute to the occurrence of the fatty liver syndrome are probably large doses of the drugs given over extended periods, pregnancy and perhaps extensive infections.

Allen and Brown[40] studied the role of the factors mentioned by Whalley _et al_[37] and Dowling and Lepper[38] in causation of liver damage and found that there was a consistent increase in the amount of fatty metamorphosis after administration of drugs even though the doses of tetracyclines given were approximately half of that reported by Schultz _et al_[34]. The role of other factors such as infection and pregnancy could not be confirmed.

Hypersensitivity reactions are uncommon with the tetracyclines, but

nevertheless they do occur. Dowling[41] reported a survey of 716 patients and showed that only 6 of the patients had a reaction of any kind, and of these, only 1 exhibited an allergic reaction. Fellner and Baer[42] reported the case of a 44 year old man who developed general symptoms of anaphylactic reaction after taking two capsules of tetracycline hydrochloride. Cases of angioneurotic edema and generalized skin lesions have also been observed[43].

Photosensitivity reactions in connection with all the tetracyclines have been reported by many investigators[44-51]. They are usually more common with CTC and DCTC. Cullen _et_ _al_[52] have reported the case of a 17 year old white female who developed "stinging and tingling" sensations on the dorsum of the hands and feet. She had been given tetracycline HCl for a period of 5 days. They pointed out that "the capacity to photosensitize is inherent in the unsaturated resonating ring structure of the molecule, although that capacity may be increased by chlorination". Fanconi syndrome, due to ingestion of outdated TC, has also been reported[53,54].

The Fanconi syndrome presents with signs of rickets in children and osteomalacia in adults. It is believed to be due to deficient reabsorption of different substances in the proximal convoluted tubules of the kidney. Because of defective renal tubular reabsorption of substances, namely glucose, amino-acids, phosphate and bicarbonate, the conditions of glucosuria, amino-aciduria, hyperphosphaturia and metabolic acidosis may appear.

MODE OF ACTION OF TETRACYCLINES

The tetracycline drugs are usually regarded as bacteriostatic and not bactericidal. In higher concentrations, they have shown to be

bactericidal in vitro[55], but such concentrations are usually not attained in the blood stream. The mode of action of none of these drugs is understood. A number of specific reactions have been described which may be involved and are summarized as follows:

(1) Inhibition of protein synthesis[56].

(2) Inhibition of nucleic acid synthesis[56].

(3) Interference with oxidative and fermentation reactions[57].

(4) Interference with oxygen consumption and general respiration[57].

(5) Inhibition of oxidative phosphorylation[58-60]. Chlortetracycline was found to inhibit oxidative phosphorylation in rabbit kidney and rat liver mitochondrial preparations.

(6) Effect on Krebs Cycle oxidations and electron transport[61].

(7) Since these drugs have affinity for di- and trivalent metals, they may interfere with many enzyme systems in mammalian and bacterial cells[62,63].

II. TETRACYCLINES AND TEETH

Between the period of 1956-1959 the first reports of tooth discoloration due to tetracycline administration was made by Shwachman and Schuster[64,65] and Rall et al[66]. Shwachman and Schuster reported that out of the 300 children examined who had been given tetracycline in doses of 10-20 mg per kilogram body weight for a period of 1 year or more, 5 percent showed discoloration of the deciduous teeth. Rall et al[66] mentioned that the tetracycline deposits in the teeth were fluorescent when viewed under ultraviolet light.

In 1961 Zegarelli and associates[67] and Wallman[68] reported that patients who have been treated with tetracycline drugs showed yellow discoloration of the teeth. Zagarelli et al[67] surveyed the records of 52

patients who were given tetracyclines for fibrocystic disease of the pancreas and found that 38 patients had discoloration of the teeth, which varied from gray to brown to black in color under ordinary light. At the same time Bevelander and his associates[69], and Gron and Johannessen[70] studied the effects of tetracycline HCl on the rat incisor. The ground sections of the teeth showed clearly represented fluorescent lines in the dentine corresponding to the period when doses of tetracycline were given. Since these lines were also visible in ordinary light, Bevelander and associates concluded that enamel and dentine was less calcified. Some fluorescence was also seen in the enamel. Microradiographs of the teeth prepared by Gron and Johannssen[70] also showed areas of weak mineralization corresponding to the lines.

Boyne and Miller[71] studied the fluorescent color intensities of OTC and CTC in the developing canines of eight mongrel dogs. Two tetracyclines in doses of 10 mg/kg body weight were injected alternately at intervals of 10 days. Examination of the ground section under U.V. light showed a characteristic yellow fluorescent pattern within the dentin, resulting from the administration of OTC. It was noted that CTC gave a deper orange pattern. They suggested that variation in characteristics of the fluorescent bands can be used for possible chronologic orientation of growth pattern in developing teeth. No inhibition of root development or calcification was noted.

Owen[72,73] studied the effect of CTC on teeth of two months old greyhound puppies. He administered 750 mg of the drug orally, daily for 6 days a week for one month. All the erupted permanent teeth were found to be pale yellow in daylight, and exhibited intense yellow fluorescence under U.V. light.

Wallman and Hilton[74] reported on fifty human infants who were given tetracycline during their first week of life for various reasons. Their conclusions were as follows:

> "The pigmentation varied according to the age of the child, in younger children the pigmentation was bright yellow and in the older ones it was brownish. In more premature babies the yellow color involved more of the incisor teeth and in some all the visible enamel was effected where pigment was severe, there was also deformity of canine and molars. In general, the larger the total dose relative to body weight, the more severe the abnormalities, the color being deeper and the enamel hypoplasia greater."

They further reported that only one child out of eight children who had oxytetracycline exhibited yellow pigmentation of the teeth. Four children had no pigmentation at all even though they received higher doses of OTC in comparison to the child mentioned above. They remarked that "tetracycline may now be the commonest cause of enamel hypoplasia in young children". They also raised the question "Are the permanent teeth affected?".

Wallman and Hilton's article was the first of the series of articles which appeared in 1962 and thereafter. More case histories and surveys regarding tooth pigmentation due to tetracyclines were published.

Porteous and Weyman[75] reported two cases in which tetracycline was given for osteitis of the maxilla and fibrocytic disease of the pancreas. In the first case drug was given continously from the age of 12 days to 6 months. At the time of examination (7 years of age) the deciduous teeth present were grey brown. None of the permanent teeth were affected. In the second case the drug was given almost continuously from the time that the patient was 11 months old. At the time of examination (8 years, 5 months) her deciduous teeth were normal in all respects but the permanent teeth were grey brown, except the tips of eight incisors

which were reasonably normal in color. These findings suggested that the teeth which were developed either before or after the administration of the drug were not affected, but only those teeth or parts of teeth being formed during therapy were discolored.

Rushton[76], Stewart[77] and Miller[78,79] took issue with the statement of Wallman and Hilton[74] that "tetracycline may now be the commonest cause of enamel hypoplasia in young children". They pointed out that enamel hypoplasia could be due to prematurity alone or disease for which the drug was given. Miller[80] mentioned that 50% of prematurely born children have been shown to have yellow teeth. This yellow pigmentation has been attributed to enamel hypoplasia of the teeth; thin enamel permits the normally yellow dentin to be seen. Such enamel hypoplasia has usually been assessed as being of neonatal origin.

Wallman and Hilton[81] then published another survey. They examined the records of 46 children whose birth weight was less than 2.5 kilograms (the criterion used for prematurity). The average gestation period was 35.1 weeks, and all had received tetracyclines for various reasons. Of this group, 32 children had normal teeth, and even though few children suffered from neonatal illnesses, the other 14 children showed varying degree of hypoplasia and pigmentation. From these observations they concluded that it was the tetracycline, rather than the illness being treated, which was directly responsible for enamel hypoplasia. Such observations have been made by many investigators.

In another group of 21 children who had been given quite heavy doses of OTC in the neonatal period, only 2 children showed abnormal teeth. One child was mentally defective and the other had kernicterus. The teeth in these 2 instances showed moderate enamel hypoplasia with slight

pigmentation. It was observed that OTC gave no pigmentation or enamel hypoplasia in comparison to TC where 46 patients of 50 showed structural defects.

As time progressed more case histories linking tetracycline drugs with hypoplasia and pigmentation were reported on both sides of the Atlantic; by Pindborg[82], Weyman and Porteous[83], Zegarelli and his associates[84] and others[85-88]. Most of the researchers agreed that there was a direct relationship between the time of administration of the drug and the part of the tooth affected. Beckelman _et al_[89] reported a case of a 5 1/2 year old girl who was given oral tetracycline for pneumonia when she was 2 weeks old. Clinical examination showed that the deciduous teeth were discolored by bands of yellow brown stains and disfigured by areas of hypoplasia of the enamel. The discoloration was present in the cervical 1/3 of the central and lateral incisors, most of the crowns of the molars and the incisal 1/2 of the cuspids - in other words, in the areas of the teeth which were developing at that time.

Zegarelli and associates[90] made interesting observations on patients suffering from cystic fibrosis of the pancreas, who had received tetracyclines. Twenty-one of the 31 patients showed distinct discoloration of two or more teeth, whereas only 11 out of 31 patients exhibited fluorescence of two or more teeth under U.V. light. They explained that among other reasons it could be due to (1) the tetracycline deposited in tooth substance may be in a form precluding fluorescence via the excitation technique used, (2) variation in sizes of teeth and thickness of overlying enamel, (3) disease or diseases may effect the developing teeth to such an extent as to prevent the deposition or fluorescence.

Except for a few studies, most of the reported observations were of

a subjective nature based on clinical cases observed in the hospitals. Harcourt _et al_[91] and Atkin _et al_[92] studied the distribution of tetracycline in teeth of patients undergoing long term treatment with tetracyclines. Macroscopically the first deciduous molars were hypoplastic and most of the enamel on the occlusal surface was worn away. Ground histologic sections under ordinary light showed faint yellow lines in the dentine towards the occlusal surface, enamel being unstained. The presence of large areas of interglobular dentine did not appear to be related to the yellow bands. The yellow bands exhibited golden yellow fluorescence under U.V. light. The dentino-enamel junction area fluoresced golden yellow, but was not continued in the enamel. No obvious fluorescence was observed in the enamel. Microradiographs confirmed a large number of interglobular areas throughout the dentine, and their presence was associated with areas of dentine not colored with tetracycline antibiotics.

The observations on absence of fluorscence in the enamel are in contrast to observation made by Owen[72,73] who mentioned that enamel of dogs teeth fluoresced following tetracycline administration. They pointed out that the effect observed by Owen could be due primarily to light scattering from the faces of the enamel rods and not the fluorescence of the interprismatic substance. In spite of the earlier reports of demonstration of inhibition of calcification in chick embryo and sand dollar larvae by Bevelander and associates[93,94], they maintained that the observed presence of large areas of interglobular dentine represented a manifestation of a systemic disease rather than interference with calcification by the drug. They speculated that the administration of the drug periodically suppressed the systemic illness, thus allowing the

normal calcification to occur, and that would explain why in some cases the calcification appeared to be normal in the region of the dentine which showed yellow fluorescence.

Harcourt[95] further mentioned that absence of the fluorescence and discoloration noted in some cases could be due to greater thickness of enamel masking any coloration in the underlying dentine. Since the demonstration by Storey[96] of incorporation of tetracyclines in the immature enamel and its subsequent masking when the enamel matures, Harcourt[97] modified his earlier views and reported the presence of fluorescence in the enamel of deciduous teeth of patients who had received large doses of tetracycline in the first few weeks of life. Microradiographs of the sections showed that fluorescent areas were hypomineralized. Subsequently, Weymen[98] confirmed these findings quite conclusively and said that "the clinical discoloration is caused by staining in the enamel and not in the dentine". In patients who have had prolonged therapy, the drug could enter the enamel on two possible occasions. Firstly, it must enter during matrix formation, and secondly, it is possible that the drug enters the enamel during maturation. She concluded that there is a possibility that there are higher concentrations of the drug in enamel than is indicated by the amount of fluorescence observed as compared to dentine.

Besides the few studies reported earlier, the data concerning prevalence of discoloration in a random sample of children is very meagre. In the most extensive study so far published, Frankel and Hawes[99] found that 35 (2.3 percent) of the 1724 school children showed tooth pigmentation typical of that caused by tetracycline. Each of the 35 children had received tetracycline therapy. They found that there existed a positive correlation between the time of administration of the tetracy-

cline drug and the distribution of the tooth discoloration. They suggested that administration of tetracycline between birth and 3 months of age would cause discoloration of the deciduous central and lateral incisors, between 3 months and 10 months of age, would affect the deciduous cuspids and molars. If the drug is administered between 10-24 months of age, then discoloration could be produced in the permanent first molars, central and lateral incisors and canines.

Krasny[100] examined 1231 second grade children and found that only 1.5 percent had tetracycline stained teeth.

Hennon[101] conducted a survey of 1707 school children between the ages of 5-11 years and reported that 60 (3.5 percent) out of 1707 revealed tetracycline pigmentation. The prevalence decreases with increasing age and no differences between the sexes were found.

In a more recent survey Brazda and Kratova[102] examined 476 Czechoslovackian children 2-15 years old who had been treated with tetracyclines. Only 0.7 percent showed discoloration of the developing teeth. They suggested that the usual dose of 25 mg per kilogram body weight administered for 7 days does not lead to such discoloration in children in this age group.

Witkop and Wolf[103] studied hypoplasia and intrinsic staining in 17 children ranging between the ages of 2½ years to 7 years. In five cases the exact dosage of tetracycline could be determined. The doses ranged from 20 to 75 mg per kilogram body weight per day. They found that severe hypoplastic defects in teeth were present in those children who had received the higher doses. All children showed yellow to brown discoloration and bright yellow fluorescence under U.V. light. They further observed that exposure to sunlight had reduced the yellow fluorescence

and teeth appeared more brown in color in older children.

Weyman and Porteous[104] substantiated the observations of Wallman and Hilton[81] and showed, in a study on post-mortem material of a 3 year 2 month old child who had received TC and CTC starting at the age of 9 weeks at different times during 3 years of illness, that in addition to discoloration and fluorescence of the deciduous teeth, the permanent tooth buds also showed vivid yellow gold fluorescence. The enamel showed mild fluorescence but they were not sure it was due to tetracycline. The dosage pattern was clearly reflected in the dentine. Since this patient's medical history bears no relationship to other reported cases of yellow discoloration, they concluded that fluorescent bands in the dentine were definitely caused by the administration of tetracyclines.

Weyman and Porteous[105] further showed that there are two types of discolorations. The first was strong yellow which fluoresced in U.V. light, whereas the second was a grey brown, which has no fluorescence. They suggested that yellow teeth erupt yellow and do not become brown as some people have indicated. The grey brown teeth usually erupted darker than normal and become even darker later on. Their findings that patients with grey brown teeth had been given CTC while those with yellow teeth had received TC sparked an interest in studying the comparative discoloration and fluorescence given by different tetracycline analogues.

Owen[106] conducted experiments in dogs to provide answers for questions raised by Wallman and Hilton and others. He attempted to find out whether permanent teeth were affected and whether all tetracyclines produced the same abnormalities.

Therapeutic doses and high doses (2-6 times the therapeutic doses) of 4 known analogues of tetracycline were administered orally to 6-7 week

old dogs for a period of 28 days. The animals were sacrificed at the end of 18 to 24 weeks. The following observations were made.

After 4 weeks of administration of therapeutic doses the deciduous teeth showed discoloration which varied from dull yellow (DCTC) to brownish (CTC) to dull white (OTC). Under U.V. light fluorescence varied from yellow (DCTC) to dull cream (CTC) to faint creamy yellow (OTC). After 2 weeks administrations with high doses the deciduous teeth showed discoloration varying from pale yellow (DCTC) to brown cream (CTC) to faint dirty cream (TC and OTC). Under U.V. light all the teeth showed yellow fluorescence.

Examination after six months of all the permanent teeth in the therapeutic dosage group showed no gross defects. Teeth showed discoloration ranging from deep yellow (DCTC) to pale yellow (CTC) to dull pale cream (TC) to whitish (OTC). In U.V. light all teeth gave yellow or orange fluorescence. Permanent teeth of dogs given high doses showed no gross defects except in the case of OTC, where 2nd and 3rd incisors showed considerable loss of enamel. All the teeth were yellow and they fluoresced deep orange (DCTC) to deep yellow (CTC, TC) and pale yellow (OTC).

Ground sections revealed no fluorescence of enamel of deciduous teeth. Permanent teeth at both dose levels showed bright fluorescence of the enamel especially that portion close to the dentino-enamel junction. Its intensity depending upon the tetracycline used.

Johnson and Mitchell[107] followed the work of Owen[106] and studied the fluorescence of rat incisors of 4 homologues of tetracycline given in therapeutic doses for 21 days. They reported no discoloration or enamel hypoplasia under ordinary light. DCTC and CTC produced equal degrees of fluorescence (yellowish) and certainly greater than that of TC and OTC.

Isben, Urist and Sognnaes[108] studied the difference in behaviour among the tetracyclines in teeth of rabbits. They injected intramuscularly 10 mg per kilogram body weight of each of the four tetracyclines in one group of animals and gave oral administration of 20 mg per kilogram body weight to the other group for a period of 30 days. Irrespective of the route of administration they found that all the teeth fluoresced brilliant yellow in the U.V. light. The intensity of fluorescence was $\text{DCTC} = \text{TC} > \text{OTC} \gg \text{CTC}$. Under ordinary light the discoloration noted was $\text{DCTC} > \text{TC} = \text{OTC} > \text{CTC}$. After exposure to sunlight for 12 hours teeth stained with CTC and OTC lost their ability to exhibit fluorescence, while teeth stained with TC and DCTC showed only faint fluorescence. All these teeth tended to become brown.

In contrast to the findings of Owen[106] and Johnson and Mitchell[107], where OTC was found to give the least discoloration and fluorescence and DCTC and CTC the most, Isben, Urist and Sognnaes[108] showed that intensity of fluorescence was $\text{DCTC} = \text{TC} > \text{OTC} \gg \text{CTC}$. Under ordinary light the discoloration noted was $\text{DCTC} > \text{TC} = \text{OTC} > \text{CTC}$. They felt that the relatively weak fluorescence of CTC stained teeth suggested that quantitatively teeth bind less CTC than other derivatives. Secondly, there might be some differences among the breakdown products of different tetracyclines. They concluded that CTC is the least likely to cause discoloration. They also did not find any hypoplastic defects in teeth at the dose level administered.

Association of different types of discoloration with various types of tetracycline was clinically investigated by Weyman[109-111] in patients who had received tetracyclines. She reported that the colors could be divided basically into 3 groups, yellow, grey brown, and brownish. Re-

view of the records of 59 patients showed that yellow teeth were associated with DCTC, TC and OTC, and grey brown teeth were associated with CTC. OTC showed the least objectionable coloration, whereas severe staining and disfiguring was always associated with CTC, TC and DCTC. These observations supported the earlier findings that, with respect to dental discoloration, OTC is the least objectionable of the 4 tetracyclines. An exception is the finding reported by Isben, Urist and Soggnnaes[108] who found CTC to be the least objectionable.

Bevelander and associates[112] studied the effect of TC on fluorescence and visible coloration of the incisor teeth of rats of different ages and reported that the width of the increment of dentin and enamel which exhibits fluorescence and yellow coloration is related to the amount of drug given. For example: administration of 10 mg per kilogram and 100 mg per kilogram resulted in a fluorescent band in dentin 5μ and 60μ wide respectively. At 10 mg per kilogram the drug was not observed in visible light whereas at a level of 100 mg per kilogram a 25μ wide bright yellow band was observed. The higher dose levels produced fluorescent and visible yellow bands which were 2-3 times as wide as the amount of dentine and enamel laid down in 24 hours. They explained that this increase in width of the fluorescent and discoloration area in teeth at higher dose levels may be due to (1) the presence of the drug in the body fluid over longer periods than previously recorded or (2) that the tetracycline is incorporated in tissues undergoing mineralization at the time of drug exposure and also in recently or incompletely mineralized tissues. They concluded that both fluorescence and visible coloration increased in extent and intensity with increase in amount of drug administration, but this increase is not strictly proportional. They further

confirmed the clinical observations made by Wallman and Hilton[74,81] of young children and reported that fluorescence and yellow coloration are more intense and more widely distributed in teeth of younger animals than in older ones. The observed differences at different age levels may be due to the presence of more incompletely mineralized (reactive) sites in the younger teeth. They concluded that the degree and extent to which enamel and dentine are discolored by the drugs is dependent on the age of the rat dosage employed.

FETAL STAINING

A number of reports of discoloration of the teeth of children have appeared in the literature from time to time. In 1954 Charles[113] reported that TC, OTC and CTC pass from the maternal circulation to the fetal tissues.

In 1960 Gibbons *et al*[114] showed DCTC crosses the placental barrier and serum levels in the fetus may reach as high as 2/3 of that in the mother's serum. They pointed out that 150 mg daily doses given 3 to 6 days before delivery produced a mother's serum level of 3.77 μg/ml and a level in the fetus of 2.47 μg/ml.

Further evidence that tetracyclines pass the placental barrier was provided by Bevelander *et al*[115] in laboratory animals and Cohlan *et al*[116] in human subjects.

Madison[117] reported a case of a pregnant woman who had taken 5 gm of tetracycline in the last 4 months of pregnancy which resulted into deposition of the drug in calcifying teeth. Without any proof he argued that if 1/4 of this dose passed through the placenta into the fetus, it would provide more than 400 mg/kg of body weight in the fetus which is more than twice the amount of 189 mg reported by Wallman and Hilton[81]

to cause pigmentation of the teeth.

Douglas[118] showed that all children born to women who had been given long term tetracycline therapy during their pregnancy showed deciduous teeth which fluoresced under U.V. light.

Similar observations have also been made by Kutscher *et al*[119], Kline *et al*[120] and many others[121-125] between 1962 and 1965. Kutscher *et al*[119] reported a case of a child 3 years 2 months old whose mother had received 20.75 gm of tetracycline at three different times, after a gestation period of 28 weeks, for premature rupture of the fetal membranes. The child did not receive any tetracycline after birth. At the time of examination all the teeth were discolored and they fluoresced under U.V. light. These authors did not discuss which parts of the teeth were affected.

Kline *et al*[120] studied nine patients whose mothers had received tetracycline during pregnancy. At the time of examination the ages of the children ranged from 18 months to 5 years. Of the nine children, seven showed positive yellow fluorescence. The mothers of these seven children had received TC or OTC or DCTC between the 5th to 8th months of the gestation period. The other two children whose mothers had received total of 21 gm of OTC and 12 gm of DCTC at the three months gestation period revealed no fluorescence of the teeth. These authors felt that the stages of gestation at which the drugs can effect the teeth is very important. Since the calcification of deciduous teeth begins during the middle of the fourth month of gestation, no discoloration is possible below this age even though the doses were heavy.

Weyman[126] reported observations on children whose mothers had had tetracyclines during the later half of pregnancy. Two children whose

mothers had TC and DCTC respectively, showed vivid yellow coloration of incisor teeth. Another child whose mother had OTC had teeth which were creamy yellow.

Toaf and Ravin[127] examined 94 children 3 to 6 years old whose mothers had been given TC and OTC during pregnancy. An average dosage of 1 gm for a period of 15 days had been administered. The results showed that none of the 47 children whose mothers had TC during a gestation period of 15-24 weeks had discolored teeth. Of 15 children exposed from the 25th to the 28th week, only one was effected. Approximately 50% of 25 children exposed from the 29th week to term had discolored teeth. The suggestion that tetracycline treatment should not be withheld from pregnant women up to the 25th week of gestation are in sharp contrast to the observations made by Kline _et al_[120] and Weyman[126] who found that deciduous teeth were affected any time after 5 months of gestation. Toaf and Ravid[127] proposed that pregnancy from the 29th week to term should be considered a definite contraindication for tetracycline therapy.

Owen[106] studied the phenomenon of placental transfer of tetracycline in a pregnant greyhound bitch. The bitch was given oral doses of tetracycline averaging 100 mg per kilogram of body weight during the last 8 days of pregnancy. Teeth and bones of the pups were studied when they were one day old and 8 weeks old.

The deciduous incisor teeth, tips of the cusps of the canine and deciduous molars both at one day and 8 weeks of age showed yellow coloration in daylight. Under U.V. light, there was bright yellow fluorescence with clear demarcation lines between the yellow and normal color of the teeth.

Histological examination of the primary teeth showed a yellow

fluorescent band of dentine adjacent to enamel. Enamel also showed a pale yellow fluorescence.

Johnson and Mitchell[107] also studied the question of placental barrier in rats. They fed rats tetracyclines in human therapeutic dosage from the time of mating until the offspring were born. Examination of the 15 day old offspring showed yellow fluorescence, but by the 21st day the yellow fluorescence had become a light pink. Microscopic sections of the 21 day old rats had little or no fluorescence.

III. TETRACYCLINES AND BONE

Since John Hunter first observed stained bones of pigs due to madder feeding, bone physiologists have searched for compounds which would stain the bone, but have no toxic effects. Many substances such as alizrin, radiocalcium and radiophosphorus have subsequently been used to label the bone and teeth to study their physiology. In 1948, a new drug was discovered, a tetracycline (CTC), which besides having wide spectrum antimicrobial activity had the property of localizing in the teeth and bones.

Even though Regna _et al_[128] and Albert[129] reported as long ago as 1951 and 1953 the avidity of the drug for heavy metal, and Andrea[130] (1956) first demonstrated the localization of tritiated tetracycline in the bones and teeth of mice, it was not until the reports of Milch, Rall and Tobie[131,132] that real interest in the use of the phenomenon of tetracycline fluorescence as a means of studying skeletal physiology began. Since the appearance of this report, tetracyclines as labelling agents have been quite extensively used in studying bone growth, bone remodelling, growing teeth, calcifying cartilage and tumor diagnosis. Accord-

ing to Frost[133], the label obtained with tetracycline drug is quite comparable to other known labelling techniques such as alizrin, trypan blue, radiocalcium, radiophosphorus, etc., and has the added advantages of simplicity, economy and effectiveness and without any disadvantages of the other agents. Very little equipment is needed to do useful work.

Milch and associates[131,132] studied the localization of TC, OTC and CTC in both human subjects and laboratory animals. Sprague Dawley rats weighing 150 gm and white rabbits weighing 1-3 kilograms were given single intraperitoneal doses of 50 mg per kilograms of body weight, of different tetracyclines. Doses ranging between 0.1 mg per kilogram to 200 mg per kilogram were also given by parenteral routes and by mouth. Animals were sacrificed at intervals from 30 minutes to 10 weeks. Human specimens were obtained either at time of surgery or at time of autopsy. The results have been summarized as below.

Following parenteral administration, a brilliant yellow gold fluorescence was observed in all tissues. In soft tissues the fluorescence was diffuse and could not be seen 6 hours after administration of the drug. However, the fluorescence persisted in bones and teeth for prolonged periods. The pattern of distribution of the drugs was completely independent of dose, sex and route of administration. Each tetracycline produced qualitatively identical results in all the animals studied. Differences in localization in bone were observed and these were due to differences in skeletal age of the animals. Minimum bone fluorescence could be detected in animals given 0.25 to 0.30 mg per kilogram of drug parenterally.

The bones of rats sacrificed 10 weeks after the injection of tetracyclines showed deposition of new bone over the previously stained bone,

thus masking the earlier fluorescence. The human samples of bone showed intense fluorescence in the area of new bone growth. The general distribution of the fluorescence was along the periosteal and endosteal surfaces of the bones. From this observation they suggested that there was a direct relationship between localization in these regions and bone blood supply.

Frost and his coworkers[133] were so enthused about this phenomenon of tetracycline fluorescence in skeletal tissues that they remarked that the findings of Milch et al[132] had ushered in a new era in the study of bone physiology. They wrote a series of papers on the physiology of human bone in vivo using tetracycline as a label. They studied and measured the biological half life of the haversian system, the time required to form a haversian system, osteoblastic activity, osteoclastic activity and the presence of osteoid seams. They also perfected the technique of studying tetracycline labelled bone materials. An account of their observations is given below[133-137].

> "The labelled bone appears as yellow stained bands, the bands being parallel to and in the substance of lamellae. Bands do not cross lamellae or cement lines. The drug deposits in mineralizing bone and cartilage and appears fixed there by subsequent mineralization."

They further said

> "The labelled band width is a direct function of duration of dosage and rate of mineralization of osteoid while intensity of fluorescence and visual yellow is a function of dosage per day. When life continues after cessation of dosage the labelled bone and calcified cartilage become buried by unlabelled bone and calcified cartilage unless appositional growth ceased during drug administration."[135]

Following the administration of the drug the tetracycline blood levels continue to rise and maximum blood concentration is reached between 3 and 4 hours. During this period all the available surfaces of

the bones such as walls of Haversian systems, lacunae, Volkman's canals, periosteal and endosteal surfaces are stained, but to a variable degree. Feather bone and very young bones are diffusely stained. Maximum intensity of the fluorophore is noticeable where the new bone is being actively laid down. Within 48 hours of cessation of the drug, the stain disappears from surfaces which are not active. The surface labelling and diffuse labelling are less firm and are readily reversible in both _in vivo_ and _in vitro_ systems. Frost _et al_[134] are of the opinion that even for permanent label a period of approximately 4 days _in vivo_ after deposition is required for the drug to be effectively immobilized in the bone.

Since the tetracycline is localized in bone along with newly deposited minerals, the tetracycline deposition reflects the pattern of mineralization. In mineralized bone the bands of tetracycline deposits remain in situ until such times as the bone resorbs or remodels. The presence of the drug in bone has been observed to be as long as 11-12 years and does not appear to diffuse throughout the biological system [136,137,144].

In regard to the types of tetracyclines and the mode of their administration, they[133] wrote that any of the four commonly available tetracyclines in therapeutic doses would produce satisfactory label in bone and other calcifying tissues. Milligram for milligram, DCTC appears to give the maximum and brightest yellow fluorescence[136]. In order to obtain satisfactory results fresh drugs should be employed as aged drugs have been observed to lose their ability to fluoresce. Oral administrations were found to give low intensity and rapid fading in rabbits and dogs, whereas intraparitoneal and intravenous administration

give most intense and permanent labels in both animals and humans.

The phenomenon of fluorescence can be used to study in vivo bone formation in regard to quantity, location and rate. Studies of bone formation can serve as indirect evidence of bone destruction, since formative and destructive processes balance. In pathologic conditions, if quantity and location of new bone formation be measured, then the comparison with findings from normals can reveal an estimate of the nature and extent of disturbance in osteoblastic activity.

Frost et al[138,139], using in vivo tetracycline labelling of active mineralizing bone, studied and measured directly the osteoblastic activity in man and reported that though the drug instantly stains 4 micron thickness of the mineralizing osteoid, on an average only 0.9 micron of new osteoid per day is formed in actively forming haversian system in adults. The initial deposits of tetracyclines are leached out when there is no more drug in the blood circulation. It takes less than 8 weeks to complete a given haversian system. They[140] further reported that a resting state of osteoid seams also occurs and these are morphologically identifiable. The seams are characterized by cessation of formation of matrix and its subsequent mineralization. Feather bone has been observed to be more or less impermeable to tetracyclines. Frost[141] is of the view that "in vivo, some diffusion barrier exists which isolate feather bone from the blood". The failure of antibiotic therapy to cure pyogenic osteomyelitis may be due to a very high impedence of diffusion in dead bone present at the site of the lesion. This diffusion impedence may impair the permeation of antibiotics into the bone[142].

Frost et al[143,144] showed that permanently fixed tetracyclines are deposited in the zone of demarcation which separates osteoid seams from

mineralized bone. Thus tetracycline label, by virtue of its fixation in the zone of demarcation, will also indicate the stage of completion of the labelled osteon at the time of drug administration. Not all the seams have been observed to take TC label[145].

Vanderhoeft and his associates[146] feel that tetracycline label is superior to Alizrin red which has been found to cause slowing of growth. When used in conjunction with microradiography of the same sections, they are complementary to each other.

Tetracyclines have also been used to study conditions like osteogenesis imperfecta[147], osteoporosis[148-150], the phenomenon of tooth eruption[151,152], orthodontic tooth movements[153], healing of dental extraction wounds[154,155], existence of periosteal appositional bone growth in the aged[156] and finally localization of tumors (refer section tetracyclines and tumor localization). Jett _et al_[147] studied with help of tetracycline label bone formation rate in two women with osteogenesis imperfecta and found that bone formation rates were more than 3 times faster than normal.

Jowsey[150], using tetracyclines and microradiographic techniques, studied growing osteons in osteoporotic and normal bone of the aged. The osteoporotic bone was compared with the normal bone in an effort to find whether reduction in bone mass seen in osteoporotic bone was the result of failure of bone formation or an increase in bone resorption. She microdissected the osteons from the rest of the section and then estimated the amount of tetracycline present in the growing osteon and the non-growing area. She found that only 31 to 62 percent of the total amount of tetracycline in the bone sample was located in the growing osteons, whereas 38 to 69 percent was associated with processes other than

growth such as surface labelling and diffuse labelling. She concluded that an estimate of the total amount of tetracycline in a piece of bone does not reflect, in true sense, the amount of new bone formation. These observations were in accord with the ideas of Frost and his group who believe that all of the physiological bone surfaces are diffusely stained to variable degrees.

Harris, Jackson and Jowsey[157] studied in vivo distribution of tetracycline in canine bone and compared the tetracycline and fluorescence with microradiographic and autoradiographic findings. They found that TC is incorporated into all the surfaces which were undergoing active mineralization and it remains there indefinitely. Fluorescence is also observed in resorption cavities and in inactive surfaces but disappears quite rapidly. Beside deposition of tetracyclines at sites of new bone growth, tetracycline was also bound firmly and diffusely to areas that were not the sites of mineralization or new matrix formation. TC was also found to be incorporated into surfaces of lacunae and haversian systems. These observations are in keeping with observations made by Frost. They concluded

> "The presence of tetracycline incorporation into the skeleton by means which are not related to new bone growth makes it impossible to estimate skeletal accretion accurately from a chemical determination of the total tetracycline content of a bone sample".

Since all the sites of active new bone formation were stained, it is evident that tetracyclines can be used as a reliable intravital stains for determining rates of accretion of bone if experiments are properly designed and controlled.

Hilton observed 2 children, less than 3 years of age, who had been given massive doses of tetracyclines. He reported the presence of a

striking yellow color in the bones. The pigment was present throughout the entire thickness of the cortex and also involved bony trabeculae of the medullary cavity. The cartilage did not appear to be affected. Under U.V. light the bones gave yellow fluorescence. On exposure to sunlight the fluorescence diminished and bones gradually became brown.

IV. TETRACYCLINES AND TUMOR LOCALIZATION

Rall and his associates[158] (1957) and Loo and his associates[159] (1957) were among the first to report that tumor tissues localize tetracycline fluorophor in quantities which can be detected under ultra violet light. Loo *et al*[159] gave tetracyclines to mice with implants of sarcoma and observed a bright yellow fluorescence at the site of the tumor, which persisted until the animals died. They further reported that 14 of 19 different experimental neoplasms also fluoresced under ultra violet light when TC, OTC or CTC were administered.

McLeay[160] studied two groups of patients with cancer. To one group (11 cases) he administered tetracyclines and the other group (42 cases) was used as a control. The experimental groups which had tetracycline showed a fluorescence in the cancer tissues, whereas the control group gave no fluorescence. Dead and necrotic tissue exhibited no fluorescence. Since some variations in fluorescent intensity were observed, the author suggested that it could be due: (i) immaturity of the tumor tissue, and (ii) variations in blood concentration of the tetracycline.

McLeay and Walske[161] studied the concentration of oxytetracycline in neoplastic tissues and actively growing normal bone. They administered OTC intravenously in dosage of 15 mg/kg of body weight for 3 or more days and time interval of 12-24 hours were allowed to elapse when tissues

were removed for tetracycline estimation. They observed that concentration of tetracycline in the malignant lesions were approximately 6 times greater than that in the normal new bone. Thus, bone malignancies will exhibit more fluorescence than the normal growing bone sites. The fluorescence persists in resected bone for a period of at least 6 months when stored at -10 degree centigrade. In view of these results, McLeay and Walske[161] proposed that the fluorescence of the malignant bone lesion may be of value to localize the extent of neoplastic tissue at the time of the surgical operation.

McLeay, Walske and Ogborn[162] further proposed that since tetracyclines have a definite affinity for neoplastic tissues, these drugs may be used as vehicles to carry a diagnostic or possibly even therapeutic agent to the tumor tissues.

Hakkinen and Hartiala[163] extended the above observations into experimentally produced ulcers in dogs, rats and guinea pigs. They found that the fluorescence was localized around the margins of the ulcers in all cases. Mustakallio[164] confirmed above observations and noted that fluorophore was confined only to the margin of the necrotic tissues, and did not extend into the surrounding tissues.

Vasser and his associates[165] studied 9 cases of carcinomas of various organs as well as non-specific ulcerative lesions of the skin, both in humans and rats. Tetracycline HCl was administered orally in doses of 500 mg twice daily for 2 days in humans. The animals were given oral tetracycline as a single dose of 100 mg/kg of body weight. Vasser confirmed the findings of Hakkinen and Hartiala[163] and Mustakallio[164] observing that fluorescence was localized in the margins of the ulcers. They further observed that tetracycline fluorophore localizes in a num-

ber of human malignant tumors and their metastases. Vasser _et al_[165] further reported that malignant cells did not show any fluorescence _per se_, but that fluorophore was localized in the debris and histiocytes present in the stromal tissues. These observations were later confirmed by Carter _et al_[170]. These observations suggested to them that presence of fluorophore might be an expression of stromal reaction favoring calcification rather than an indication of malignancy.

The intensity of fluorescence is dependent upon the dosage of the drug and the rate of growth of the tumor; in other words the greater fluorescence was associated with tumors which were more anaplastic[160,166].

Milch, Tobie and Robinson[167] studied the localization of tetracycline in skeletal neoplasms in 14 patients. They found that tetracycline was localized only in those areas where matrix calcification was observed. Calcified cartilage in tumors also showed fluorescence which was not detected in the epiphyses of normal experimental animals.

Bailey and Levin[168] studied seven patients with bone malignancies to determine what clinical significance tetracycline localization in tumors tissues might have in treatment of malignant tumors of the bone. They administered tetracyclines for a period of 2 days to those patients in whom bone tumors were strongly suspected. The period of 48 hours was allowed to elapse to ensure that there was no tetracycline remaining in the blood. Examination of the tumor, after surgical exposure of it, revealed a bright yellow fluorescent localized area. They further observed that tumors did not show uniform affinity for TC and that only well differentiated areas of tumors bone formation show significant amounts of tetracycline localization. Poorly differentiated fibrosarcomas showed no fluorescence at all. They proposed that gross fluorescence can be

helpful in delineating soft tissues from the invasive neoplastic tissue at the time of surgery. The use of tetracyclines as markers in the operating room has also been suggested by others. Since dead bone does not take up the tetracycline, Hattner and Frost[169] have suggested that these drugs may be helpful to demonstrate the vitality of major fracture fragments. Tapp et al[170] studied the uptake and retention of parentally administered tetracyclines in different experimental tumors (squamous tumors, ovarian tumors and mammary tumors). They found that irrespective of the degree of differentiation of the tumors tissues, tetracycline fluorescence was mostly localized in the cytoplasm of the necrotic tumor cells and other debris. Very little TC was taken up by the stroma. The living tumor cells also took up the fluorescence, but did not retain it more than 24 hours.

Because the tetracycline drugs have a prediliction to concentrate in altered neoplastic tissues, Yesner[171] and others[172,179] used them in diagnosis of lung cancer by examining exfoliated tumor cells in the sputum. Hiduchenko[172] found that of 19 patients with lung cancer, 16 showed positive results, whereas 3 out of 22 benign lesions were positive. She concluded that it was not a reliable screening test.

Klinger and Katz[173] applied this phenomenon of fluorescence to diagnose cases of gastric carcinomas. They fed tetracycline orally for a period of six days and on the seventh day examined gastric sediments. Positive results were obtained in 95 percent of the cases. None of the controls (41 cases) showed any fluorescence.

Kantor[174] and Berk and Kantor[175] in a study to differentiate benign and malignant gastric lesions followed the work of Klinger and Katz[173]. DCTC was given in a dose of 150 mg four times daily for 5 consecutive

days. After an interval of 30 hours from the administration of the last dose, observations were made on gastric sediments. The results showed that invariably fluorescence was present in all 10 patients with cancer and 2 patients with benign gastric ulcers showing mucosal atypism. Only one out of 46 patients with normal stomach gave false positive test. In another group of 45 patients, they found 80 percent of the malignant lesions were positive and approximately 5 percent of the patients with various benign lesions also showed positive results. The authors concluded that this method is quite useful to differentiate malignant from benign gastric lesions. Similar results were also obtained by Carter et al[176] in Colon washings where 6 out of 7 cases of carcinoma and only 1 out of 9 cases of benign lesions showed fluorescence.

Sandlow, Allen and Necheles[177] studied the reliability of the gastric lavage exfoliative cytology in 130 patients. In contrast to previous studies where 5 days routine of drug administration was followed, they found that oral dosage of 500 mg 4 times daily for 2 days was sufficient for the drug to localize in the malignant cells. As recommended, examination of the gastric sediment was made after a period of 30 to 36 hours from the termination of the last dose. The results showed that tetracycline fluorescence was present in all the 25 patients known to have gastric malignancy. Of the 30 patients having benign lesions, only 7 percent gave false positive. In control group of 25 patients, the test was negative in 22 patients and positive in 3 patients. Based on these observations, they concluded that it is a fairly reliable and simple technique for detection of the gastric malignancy.

Since many investigations have used systemic tetracycline and observed fluorescence in internal carcinomas, Vasser et al[165] reported two

patients with carcinomas of the skin, which showed intense fluorescence 48 to 72 hours after administration of tetracycline. The gross fluorescence persisted for 21 days.

Lipkin[178] used the phenomenon of fluorescence to devise a technique to evaluate the malignant potential of the skin tumors. He painted a test solution of 1 percent DCTC and 0.1 percent cyanocobalamine in the suspected tumor and then observed it under ultraviolet light when it was dry. The whole painted area glowed with bright yellow green fluorescence. Then 4.9 percent solution of TCA acid was applied to the area. The fluorescence was immediately quenched and if it did not reappear in 10 seconds, then the lesion was usually benign. If it persisted for 10 seconds then the lesion was malignant. Using this test they found that 68 of 75 tumors gave positive results in comparison to 191 benign tumors tested where only 5 showed false positive results.

Donsky[179] evaluated the systemic administration of TC and DCTC as a screening test for detecting malignancy in 33 patients. Of the 14 known malignant lesions 13 tests were positive; whereas out of 17 benign lesions only one test gave a false positive. One of the two patients with Bowen's disease showed positive fluorescence. They further observed that fluorescence was mainly restricted to the necrotic or granulomatous portions of the tumor. DCTC was found to fluoresce more intensely than TC. In general, the intensity of the fluorescence was evenly distributed. He felt that systemic administration of the tetracycline can be used for the reliable detection of squamous cell carcinoma of the skin.

Aberle[180] presented a critical review regarding the effectiveness of tetracycline fluorescence tests in diagnosing malignant lesions. He

studied 21 cases with gastric lesions according to the method proposed by Klinger and Katz[173] and Berk and Kantor[175]. Of the 19 known benign lesions, 7 gave false positive, whereas of 2 known malignant lesions only one gave positive result. In a critical review of the work of others he observed that:

> "It is to be noted, however, that McLeay[160] who detected fluorescence in each of 11 surgically removed tumors, used 4 times the normal dose of tetracycline, and that Phillips _et al_[166] saw no yellow fluorescence in 12 of 35 cancer specimens. Vasser _et al_[165] observed fluorescence in each of 9 malignant tumors, but also saw it in 2 of 5 benign skin ulcers. In view of these results, and of our observations, we are forced to reserve judgment concerning the 94% accuracy of the tetracycline fluorescence test of gastric lavage sediments, as reported by Klinger and Katz[173] and its 100% accuracy as reported by Berk and Kantor[175]." He finally concludes that "all that fluoresces is not tetracycline, nor is it necessarily carcinoma!"

In response to the comments of Aberle[180], Berk[181] showed in another series of patients, that of 38 patients with gastric cancer 87 percent gave positive results. Of 140 cases with benign lesions or normal stomach only 4 percent gave false positives. He admitted that failure to observe fluorescence does not mean the lesions could not be malignant or vice versa; if observations are based on larger numbers, the degree of accuracy would seem to be of an acceptably high order. He further wrote that despite its admitted falliability, the test, if properly performed, was a valuable adjunctive to diagnosis.

In spite of the controversy regarding their reliability and effectiveness, more reports continued to be published both in favour and against the technique.

Riley[182] studied the tetracycline - induced fluorescence of implanted adenocarcinoma of the lung in syrian hamsters. Of the 30 tumors studied, 26 showed positive results. Contrary to many other reports they

did not find any necrosis, infection or inflammation. The golden yellow fluorescence was found to localize within the tumor cells. They further observed that the induced fluorescence was present only when microcalcification within the tumor cell was present. The removal of the calcium with EDTA also removed the fluorescence from the tissue.

Cabrera *et al*[183] studying the phenomenon of fluorescence in several human tumors showed that in cases of primary carcinoma of the colon and oral cavity tetracycline does not specifically bind to tumor tissue but also attaches itself to the surrounding noncancerous tissues. In cases of primary carcinoma of the breast, metastatic carcinoma of the lung in the kidney and carcinoma of the colon in the abdominal wall, the localization of fluorescence was found to be specific, and was observed mainly in the cytoplasm of the cells.

Cummins *et al*[184] studied gastric sediments with the tetracycline fluorescence test and routine gastric cytology. Of 25 patients with confirmed gastric malignancy, 56 percent failed to show any definite fluorescence, whereas gastric cytology indicated the correct diagnosis in 64 percent. Of 194 patients without gastric malignancy, only 5 showed false positive fluorescence. They concluded that fluorescent tests are unreliable and that the observed variance in results obtained by different investigators are probably due to errors of interpretation of whether or not fluorescence is present.

Ackerman and McFee[185] concurred with the conclusion of Cummins *et al*[184] and observed that there is no pattern to predict the presence or lack of fluorescence in the specimens. Their examination of tetracycline-induced fluorescence in transplanted tumor in mice and hamsters gave very unreliable results. In cases where fluorescence could be observed

it was present in non-cancerous tissues, particularly in necrotic, inflammed, calcific lesions.

Aberle[186] pointed out that 13 of 53 specimens of gastric sediments from patients who were given no tetracycline at all showed positive fluorescence. Six of 15 benign gastric ulcers and one of the 2 gastric carcinoma showed positive fluorescence.

Sandlow _et al_[187] presented additional data and showed that 53 of 55 patients with proven cancer of the stomach gave positive tests, whereas of 55 normal persons there were only four who gave false positives. From 75 patients with benign gastric ulcers, three false positive results were obtained. Sandlow _et al_[187], pointed out that discouraging opinions regarding the specificity and reliability of this technique is to a large part due to studies conducted on small groups of patients, while encouraging reports are based on studies of larger numbers of patients. In more recent experiments, Burton and Cunliffe[188], Klass[189] and Herrera and his associates[190] have studied the reliability of this technique.

Burton and Cunliffe[188] assessed the value of tetracycline versus routine cytological technique in the detection of malignant lesions. They studied 175 specimens of sputum, pleural fluid, ascites fluid and gastric and duodenal juices. Of the 175 specimens, 37 were from proven malignant lesions and the remainder (138 specimens) from nonmalignant lesions. They reported that

> "tetracycline fluorescence detected 73% of the malignant cases and cytology 37%. In the nonmalignant series tetracycline studies were correct in all but 5.1% and cytology in all but 2.8%. Statistical analysis shows that tetracycline is more likely to give correct results than cytology when large numbers are examined and it will certainly give false negative results."

By combining both techniques 84% of the malignant lesions can be detected.

Klass[189] also compared the two techniques on gastric sediments and remarked that the major factor responsible for divergent results appears to be the ability of individual observers to discriminate reliably between the yellow color of other materials (bile sediments, certain cleansing compounds) encountered in gastric sediments and the correct tint of yellow seen with tetracycline. These views are in keeping with the observations of Aberle[180].

Herrera et al[190] evaluated the tetracycline fluorescent test in diagnosis of gastric carcinoma in 120 patients. They reported that incidence of false positive in 50 normal patients who had no tetracycline was 15 percent whereas in a group of 39 patients without malignant neoplasia only 54.9% were positive. Based on these observations they concluded that this test is not a satisfactory method to make differential diagnosis between benign and malignant lesions.

TETRACYCLINE BINDING IN TUMOR TISSUE

In view of the reported deposition of tetracycline in tumor tissue, Loo et al[159] studied the chemistry of the deposited fluorophore in mouse tumor tissue. They found that the fluorophore was unchanged in the tissue, as had been suspected by others. The fluorophore existed in the tissue in the form of a complex formed by the parent compound and a peptide. In acid medium this compound is readily dissociated into its respective components. This concept was further substantiated by Lacko et al[191] who claimed that CTC interacts with β lipoprotein of the blood serum in the presence of calcium ions. Studies by du Buy and Showacre[192]

and Pamuken et al[193] further strengthened the above belief by demonstrating the presence of fluorophore in mitochondria which are known to be exceedingly rich in lipoprotein. In a tissue culture system, Hooker[194] observed minimum fluorescence in the cytoplasm of malignant tumor cells, whereas in benign cells the most of the fluorescence was observed in the cytoplasm. Large concentrations of fluorophore were present in the nucleoli, presumably associated with RNA. This observation concurs with the statements of Von Bertalanaffy[195] who notes that malignant cells have a high concentration of RNA and DNA which have a great affinity for fluorescent dyes.

Titus et al[196] studied the EDTA extracts of tetracycline-induced mouse tumors. They reported that localization of fluorophore involves a metal-tetracycline bond. McLeay[160] without presenting any experimental evidence, thought that tetracycline binds to the cancer tissue probably through an enzymatic factor in the metabolism.

The constant finding of the presence of fluorescence along the margins of experimentally induced ulcers, led Hakkinen and Hartiala[163] to believe that tetracycline localization is probably due to some change in the degree of polymerization of the ground substance surrounding the ulcerated tissue. Vasser et al[165] noted that this could not be the case because the observed fluorescence was limited to ulcer margins with apparent localization in the region of necrotic debris rather than the area of regenerative tissue proliferation. This was substantiated by their finding in experimental animals in which they did not observe any fluorescence in areas surrounding absorbable gelatine sponge implants where similar changes in ground substance polymerization occurs.

Vasser et al[165] further stated that since some fluorescence has been observed in nonspecific ulcerations it is possible that tetracycline

may localize in the neoplastic stroma as a result of tissue destruction, repair and marked histocytic activity. Though the mechanism of tetracycline localization is unknown, they postulated.

> "...that the sequence of events may be indicated by transudation or exudation of free and protein-bound tetracycline into areas of tissue activity. Owing to some unknown local biological conditions such as polypeptide complex linking or calcium metabolism, there are a local binding and precipitation of tetracycline in the areas."

Further evidence that tetracycline is localized in the necrotic cytoplasm of the cells is given by Burton et al[188] and others[163,164,170, 179,185]. In tissue cultures of rabbit kidney and HeLa cells Burton et al[188] have observed both intracellular and extracellular fluorescence, with extracellular fluorescence more pronounced in the most anaplastic HeLa cultures. They further showed that intracellular fluorescence is related to necrosis, a finding in keeping with the finding of Machado et al[197] who found fluorescence in experimentally induced sarcomas only when there was some cytological evidence of necrosis. Tapp et al[170] also confirmed these observations and showed that fluorescence is retained for a period of one month in contrast to uptake and retention in living tumor cells where fluorescence disappears in 24 to 48 hours.

Milch et al[13,167] have shown that tetracycline may form a complex with ionic calcium and also with macromolecules. They have shown that tetracycline is localized in those areas where matrix calcification is observed. Riley[182], contrary to the observation of others, showed that there was no necrosis of the cells associated with fluorescence. By microradiographic technique and extractions with EDTA they showed that induced fluorescence was present only when microcalcification within the tumor cell was present. Ackerman et al[185] also showed that fluorescence was associated with necrotic, inflammed, calcific lesions.

METHODS AND MATERIALS

With the exception of demthylchlortetracycline all the drugs have the same recommended therapeutic doses. The range of these doses is so wide that it is very difficult to compute a dose level which would be called a normal standard dose. As the drugs are most commonly employed for a wide range of microbial infections ranging from mild to severe, acute to chronic, the doses required in such conditions would vary considerably. Secondly, some species differences might be involved which would further complicate the picture of selection of the normal dose. Keeping these variations in mind, the highest doses usually recommended by the manufacturers for children have been taken as the normal standard dose. The recommended doses vary between 22 to 44 mg/kg body weight per day except that for demethylchlortetracycline which ranges between 6.6 to 13.2 mg/kg per day. Thus 10 mg/kg for demethlychlortetracycline and 40 mg/kg body weight for the other three tetracycline drugs were taken as normal standard doses for this study. Five dose levels (2.5, 10, 20, 50, 80 mg/kg) were employed for demethylchlortetracycline and four dose levels (10, 40, 80, 200 mg/kg) for the others. These drugs were tested as shown in Table 1.

TABLE 1

SEQUENCE OF INJECTION, DOSAGES AND TETRACYCLINES USED

Drugs	Number of Groups	Total Number of Rats	Dose Levels (mg/kg)
Tetracycline	4	16	10,40,80,200
Oxytetracycline	4	16	10,40,80,200
Chlortetracycline	4	16	10,40,80,200
Demethylchlor-tetracycline	5	20	2.5,10,20,50,80

Commercial preparations were found to be unsuitable for the drug solutions for injection in the concentrations required in some of the experiments, because of the presence of large amounts of ascorbic acid used for buffering purposes. The pure samples of the drugs were obtained directly from the manufacturers. The name of the drugs, their brand name and source of supply are listed as follows:

Drugs	Source and Brand Name
1. Tetracycline HCl	Chas. Pfizer and Co. (Tetracyn)
2. Oxytetracycline HCl	Chas. Pfizer and Co. (Terramycin)
3. Chlortetracycline HCl	Lederle Laboratories Division, American Cyanamid Co. (Aureomycin)
4. Demethylchlortetracycline HCl	Lederle Laboratories Division American Cyanamid Co. (Declomycin)

PREPARATION OF DRUGS

With the exception of chlortetracycline, which loses most of its activity in 24 to 48 hours, the other drugs are fairly stable in solution for 3 to 7 days under refrigeration. When establishing conditions for the six day periods (over which time the injections were performed) the drugs were put into solution at different times. The drugs were weighed with a gram-o-matic analytical balance, and half an hour prior to the injections, the required amount of physiologic saline solution was added. Since the injections were repeated every 24 hours, the maximum storage period for any drug was not more than 48 hours. The concentrations of the drugs in solution were adjusted in such a manner that 0.3 ml of the solution contained the required dose to be administered to a 100 gm rat. The calculated doses and the details of the preparation of solutions of the tetracyclines are given in Table II.

TABLE II

CALCULATION OF DOSES AND PREPARATION OF TETRACYCLINE DRUGS

Calculated dose per kilogram weight mg	Required dose for 100 gm rat mg	Amount of drug weighed mg	Amount of saline added cc	Amount of drug present per 0-3cc mg
2.5*	0.25	6.25	7.5	0.25
10.0	1.0	25.0	7.5	0.25
20.0*	2.0	50.0	7.5	2.0
40.0	4.0	100.0	7.5	4.0
50.0*	5.0	125.0	7.5	5.0
80.0	8.0	200.0	7.5	8.0
200.0	20.0	500.0	7.5	20.0

*These doses were employed only for Demethylchlor-tetracycline.

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats were used in this study. The animals were housed in 4 to 5 large cages and fed on normal diet without any restriction and given tap water ad libitum. When the animals were between 85-90 gm in weight they were divided into nineteen groups with 4 in each. The animals were arranged in such a way that the mean weights of all the groups were 90 gm, plus or minus 2 gm. Of nineteen groups, 2 groups were used as controls. All the animals were weighed once daily beginning five days prior to the injection period and continuing until they were sacrificed. On the fifth day, when the weights of the animals were approximately 125 gm, they were injected, intraperitonially, once daily for six days. The injections were made with 1 cc hypodermic glass syr-

inge using a 25 gauge stainless steel needle. To maintain the required dose, the doses were adjusted on each day according to the body weight. At the end of the experiment (total time 25 days) i.e. two weeks after the last injection, the animals were sacrificed under chloroform inhalation anesthesia. Gross post mortem observations of liver and kidney with regard to color, consistency, size and shape were made of 2 animals in each group. The animals were wrapped into packing paper and stored in deep freeze until the time their bones and their teeth were taken out.

RECOVERY OF BONES AND TEETH

Since a large number of animals was used in this study, a method proposed by Savchuck[198] (1959) to free the soft tissue from the bones and jaws of the animal was employed. It involved autoclaving* the rats for 8 to 10 minutes at 15 lb/in^2 pressure. The feasibility of the method was checked to ensure that the drugs were not dispersed or otherwise altered during the procedure by comparing with animals cleaned by hand[199].

As soon as the animals were taken out of the autoclave all the long bones of the extremities as well as the upper and lower jaws were carefully dissected out and freed of easily removed soft tissues by a blunt scalpel. The partially cleaned bones and jaws were then washed in distilled water and transferred to wide-mouthed bottles containing 50 ml of neutral buffered formaline wherein they remained for a 24 hour period. This procedure facilitated the cleaning of the muscle and ligament attachments especially from the condylar regions. The bones and jaws were stored in 10 percent formaline solutions for subsequent observations.

In order to assess the effect, if any, of the four drugs on general

*Speed Clave 777 Wilmot Castle Co. Rochester, N.Y., U.S.A.

body growth, the growth curves of the animals were plotted. To study the effect on the bones, the femora and humeri of the animals were subjected to a series of measurements and analyses.

A. 1. General Body Growth Data

From the original data the arithmetic mean body weight of each group for an experimental period of 25 days was calculated. A casual examination of the data indicated that inconsistant increases or decreases in weight occurred in the animals during the period when drugs were injected. These fluctuations were most evident in those groups where the highest dose levels of the drugs were employed. To show the basic trend, which was slightly obscured by day to day fluctuations in weight, the growth data in all the groups were "three-point smoothed" by applying the technique of Boyd[200]. The technique of three-point smoothing has been illustrated in Table III; and the effect of the application of such a technique are shown in Figure I. The original weights of day one, two, and three were averaged and the mean thus obtained was considered the corrected value for day two. In the second step the original value of the day two, three and four were averaged and considered correct value for day three. Subsequent steps were thus performed and new corrected values, three point smoothed once, were obtained. The same procedure was repeated on this new corrected data. Essentially the technique tends to correct any random daily fluctuations if present. The three point smoothed values of the weights thus obtained were plotted on the abscissa against time on the ordinate and growth curves were drawn to show the comparative effect of the drugs on animal growth at similar or different dose levels.

2. Physical Measurements (Femur and Humerus)

(a) Length - Instead of measurements being made directly on bones, both femora and both humeri of an individual animal were first x-rayed on Kodak ultra-speed occlusalfilms and then measurements in millimeters (± 0.01 mm) were made directly on the films. Steps were taken to ensure a reproducible geometry; i.e. placement of the bones on the film and under the x-ray tube in subsequent exposures. The x-ray machine* was operated at 30 KVP, 5MA, at a target-film distance of 20½ inches. Seven seconds exposure time was found to be the best to obtain good bone outline and image density. The 20½ inches target-film distance, the close proximity of the bones to the film and the use of a 3/8 inch aperture in a lead diaphragm minimized the chances, if any, of image enlargement. No measureable differences were observed when the measurements made on the x-ray films were compared with a few selective measurements made directly on the bones. The marking of the reference points on the film and the distances measured are shown in Figure 2.

(b) Weight - In order to calculate the volume and density of the bones, the femora and humeri of all the animals were first weighed in triple distilled water and than in air. The technique of the weighing procedure employed is described below.

The bones were taken out of 10 percent formaline solution and washed in running water for 6 to 8 hours. They were kept in distilled water throughout the weighing procedures. A Gram-o-matic balance, with a built-in metallic hook hanging over the weighing pan was found to be the most versatile and convenient instrument. A platform was made across the pan on which an empty bottle or a bottle containing water could be

*G.E. X-ray Unit

placed. In order that the bones could be suspended from the balance, two loops were bent on either end of 2½ inch long guage stainless steel wire. One loop received the bone and the other loop was attached to the hook of the balance (Fig. 3).

First, the weight of each bone in water was recorded. Before the weighing was repeated in air each bone was shaken approximately ten times in the air with jerky motion of the elbow. This procedure was found to be more reliable for removing droplets of water adhering to the surface of the bones than soaking the water droplets off with filter paper. Further, to minimize the drying of the bones during weighing in air, the bone was lowered into an empty bottle.

(c) Volume - Was determined by water displacement method (Archimedes principle) Volume (Cm^3) = Weight of the bone in air minus weight of the bone in water.

(d) Density - Was calculated from wet weight of the bone and its volume. Density = $\frac{\text{wet weight of the bone (gm)}}{\text{volume (cm}^3\text{)}}$

To check the reliability of the above procedure as a basis of the calculation of volume and density, forty measurements were repeated after an interval of two weeks. Volume and density calculation were made from the second set of data. The maximum differences observed were not more than 7 percent for an individual bone; when mean values for the group were considered the differences were approximately 3 percent. In 80 percent of the instances the calculated values for individual bones increased 1 to 3 percent from the original measurements (Table IV). On the basis of these findings the method for determining the volume of the bones was deemed to be satisfactory.

3. Mineral Content of Bones

(a) Organic and Inorganic Constituents - Originally it was planned to determine the ash weights and calcium and phosphorus contents of all femora and humeri. However, on conclusion of the weighings which took about 18 days, it was observed that the bones had slightly decomposed. In order to control the decomposition, it was thought desirable to transfer them back to 10 percent buffered neutral formaline until such time as the chemical analysis could be made. Further observations made after 3 to 4 days after this transfer showed that all the bones including those in the control group were covered with small crystals. In subsequent days (2 weeks) the bones had partially decalcified, as they could be slightly deformed.

In view of the above developments the scheme of the experiment was revised and modified. Instead of ashing the bones to determine the total inorganic constituents and then computing the organic matter, it was thought appropriate to first decalcify the bones completely and then determine the wet weight of the residual. From the wet weight data of the bones already known, the amount of the inorganic constituents then could be calculated.

Decalcification Procedure

Decalcifying solutions were prepared as follows:

A. Sodium citrate* 50 gm dissolved in 250 ml of distilled water.

B. Formic Acid* 90 percent diluted to 45 percent (v/v) with distilled water.

Solutions A and B were stored separately and were mixed in equal parts just prior to use.

The bones (2 femora and 2 humeri) were transferred into wide mouth

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*Fisher

glass bottles. Immediately 75 ml of the decalcifying solution (citric acid-formic acid solution) was poured into the bottles and left for a period of ten days. The solutions were shaken in the bottles 4 to 5 times a day to enhance the decalcification procedure. After the first ten day period the solutions were changed twice, using 50 ml of decalcifying solution at intervals of ten days each. At the end of thirty days the extent of the decalcification was tested by taking the radiographs of the bones using 30 K V P, 5 M A, 20½" target to film distance and 7 second exposure time as mentioned earlier. The bone density was then compared with the density of the undecalcified bone.

A thirty day period was found sufficient to ensure complete decalcification of the bones. The x-ray observations were further confirmed by qualitative determination of calcium in the 24 hour washings of bones in small volumes of decalcifying solutions.

B. Fluorescent - Color Intensity of Bone and Teeth

Three methods were applied in this study to assess the type and amount of colored fluorescence displayed by bones and teeth.

(1) Gross assessment (subjective evaluation)

(2) Photometric measurements

(3) Histological examination of ground sections of teeth

Preliminary observations indicated that the intensity of colored fluorescence changed to a certain extent when the surface of the bones became dry. To control this variable, the bones were kept submerged in water while the observations and measurements were made. Further to remove the bias inherent in human judgment and day to day variations in color intensity interpretations, bones of only one animal from each group were analysed on one particular day. The procedure was repeated on other

animals. The data thus obtained on four animals were tabulated and a table showing average values was made.

1. Gross Assessment (subjective evaluation)

Two femora and two humeri from each animal were arranged in numbered glass petri dishes. The dishes were then arranged and rearranged in descending order from maximum to minimum discoloration noted by the naked eye through a magnifying glass under white fluorescent light. The scores for the extent of discoloration were made ranging between 6+ (maximum) and 1+ (minimum). The same dishes were then viewed under U.V. light in the darkroom and rearranged in order of maximum-minimum fluorescence. The fluorescent color was noted and the degree of fluorescence was recorded 10+ for maximum and 1+ for minimum fluorescence. Autofluorescence was graded as zero.

The above assessment was later repeated with mandibular and premaxillary bones. The mandibular and premaxillary bones were later rearranged from maximum to minimum discoloration and fluorescence on the basis of observation of the teeth only. The scores for discoloration and fluorescence of the teeth were made ranging between 7+ and 1+ because the range of intensity of coloration was narrower than in the case of bones.

2. Photometric Measurements

Photometric measurements were made on the femora and humeri and mandibles of each animal. A photovolt light meter equipped with a photo cell sensitive to light between the wave lengths of 375 to 650 nm was employed. The apparatus is shown in Figure 4.

In order to ensure a reproducible geometry in regard to placement of bones below the microscope a special rectangular plastic tray 1½" x

2½" was constructed. Grid lines, one in the centre and two on either side, were marked so that the distance between two adjacent lines accommodated the field of observation of Wild's dissecting microscope equipped with 0.6X objective and 10X eyepiece lenses. The right femur and humerus were positioned in one half of the tray between two grid lines and the left femur and humerus were placed in the other half of the tray. The bones were arranged with their posterior surfaces facing upwards. Two small depressions in the plastic tray were made in the distal condylar region to stabilize the positioning and subsequent placement of the bones. The full length of a femur could not be accommodated in the field of observation of the microscope, thus the ends of the femur were excluded from the view. Only two bones could be viewed at one time under the microscopic field. The bones were kept submerged in distilled water while the measurements were being made. It was found in earlier experiments that the photometric measurements tended to vary with changes in thickness of the water layer in the plastic tray. Thus, each time the bones were arranged in the trays 9 ml of distilled water was placed with a glass syringe. After the measurements were made on the right side, the tray was moved and the left side was brought below the microscopic field. The bones were placed in focus and the photo cell search unit was placed on the eyepiece. A shield was constructed to prevent the entry of stray ultra violet radiation into the photo cell. The U.V. radiation (3660μ) was directed on the bones at an angle of 45° from a distance of approximately 10 inches (Figure 4). The volt meter readings in percent units were recorded in multiples of 10.

The above procedure was repeated with the right and left mandibular bones. Both of these bones could be accommodated in the viewing field

of the microscope. In order to cut down the ultra violet radiation reaching the photo cell, a yellow gelatin filter Kl (6) was used over the objective lens. The maximum transmittence of the filter is at 580 nm, thus the photo cell registered only the visible part of the light spectrum.

Ultraviolet Photography

After the subjective evaluation of the discoloration and fluorescence had been made, the bones and teeth were photographed both in ordinary light and in ultraviolet light. The apparatus used for ultra violet photography is illustrated in Figure 5. It consists of a camera mounted on a stand and two ultraviolet lamps incident at approximately 45° to the specimens at a distance of 10 inches. Since the response of the photographic emulsion to ultraviolet light is different from the response of the eye to ultraviolet light, the method of taking photographs was standardized. Taking f=8 (lens aperture) as fixed, the time and combination of the filters required to reduce the excessive blue of the light spectrum was standardized. It was realized that the exposure time of the film would vary according to the intensity of fluorescence shown by the bones and teeth, but in view of the fact that many different groups with varying intensities were involved in this study one exposure time was given in all cases, so that the results could be compared. From the results of the preliminary experiments an aperture of f=8, a Kodak Wratten filter Kl (6) with maximum transmittence of light at 500 nm wave lengths in conjunction with an Ansco filter No. 17, an exposure time of 3 seconds and film target distance of 24 inches was found to be the best combination. In cases where the distance between the lens and the specimen had to be increased to accommodate the specimens (such as mandibles),

the exposure time of 3 seconds was still maintained.

3. Histological Examination of Ground Sections of Teeth

Preparations of Teeth

The right upper incisor tooth from one animal in each group was used to prepare the ground sections. The technique of preparing the sections is as follows:

(a) Dehydration of teeth - The teeth were taken out of the storage bottles (10 percent neutral formaline) and washed in running water for 15 to 30 minutes, dried on the filter paper and then dehydrated by successive immersions in 20 ml of 70%, 85%, 95% and 98% ethyl alcohol. The specimens were left in each concentration for a 24 hour period.

(b) Embedding - After dehydration the teeth were air dried and soaked in 10 ml of catalysed purified styrene in the refrigerator. The styrene penetrates the tissues and makes them more translucent and also it binds with the tooth surface more intimately than other embedding plastics. After 24 hours the teeth were transferred to a mixture of equal parts of styrene and bioplastic for another period of 24 hours when the teeth were removed from the mixture, wiped with filter paper and then embedded in a thermo-hardening liquid plastic (Ward's "Bioplastic") in small rectangular aluminum foil boats. After the initial setting of the bioplastic, the boats were transferred into a bulb heated oven for a period of 48 hours for final hardening of the plastic. The plastic blocks thus obtained were trimmed with a sandpaper disc mounted on a dental lathe and finally polished with powdered pumice. The plastic was clear and the embedded tooth could be seen clearly. A pencil line was marked on the plastic block at a right angle to a tangent drawn from the convex surface of the tooth close to the tip of the crest of the al-

veolar bone. This line served as a guide to an approximate plane for cutting the section.

(c) Sectioning of Teeth - The bioplastic block containing a tooth was mounted on a Plexiglas sheet with softened tracing compound in such a way that the pencil line on the block was perpendicular to the edge of the Plexiglas plate. The Plexiglas plate in turn was then fastened to the fixed mounting block of a Gillings sectioning machine (Figure 6) with two screws. The proposed plane of sectioning of the tooth was thus roughly parallel with the diamond cutting wheel.

With respect to the claim made by the manufacturer regarding the uniformity in thickness of the serial sections, it was found in early trial experiments that no two serial sections had the same thickness, when all the settings on the machine were kept constant. Besides, the sections were not planoparallel, i.e., the thickness of the embedding plastic varied at different locations.

The sections were cut at a thickness of approximately 125 micron. Theoretically, movement of 81 subunits on the measuring gauge dial should give the sections a thickness close to 100 microns, but in practice it was not achieved. In order to get sections somewhere between 90-150 microns the dial was moved 90 subunits. From three to five sections were obtained from each tooth. The cut sections were measured with a micrometer and then brought to final thickness of approximately 100-110 microns by hand grinding on a ground glass slab with pumice powder. The sections were then mounted on glass slides temporarily with glycerine for subsequent microscopic examination and later microradiography. It was also observed that if the cut sections were stored in water for a few hours they tended to warp, presumably due to strain released in the

plastic subsequent to cutting. To minimize this tendency, the pressing of the sections between two glycerine wet glass slides was found to be very helpful.

Ultraviolet Light Microscopy

Ultraviolet light microscopy was used to confirm and enlarge on the impressions gained by subjective evaluation of bone and teeth in regard to fluorescent color intensities seen. A Leitz Dialux-Pol microscope equipped with Zernike phase contrast fluorescent condenser 402 b was employed in the present study. The complete assembly, including the filters used, is shown in Figure 7. The U.V. light source consisted of an Osram HBO 200W high pressure mercury vapour lamp. It was selected as the most suitable among light sources with high luminous density in the 366 nm U.V. wave length.

Filters

In the optical path extending from the U.V. lamp to the condenser the following four types of filters were always used:

(1) and (2) were 2 mm UG1 U.V. fluorescence filters (or exciter filters). These filters block visible light and transmit the fluorescence-exciting U.V. light. The transmission curve of the 2 mm UG1 fluorescence filter shows a certain amount of transmission in the blue and red spectral regions. The blue transmission is reduced when two of the filters are used together (2 + 2 mm UG1).

(3) was a red suppression filter, 4 mm BG38 filter. It was employed to reduce the residual red transmission.

(4) was a heat absorbing filter, a 2 mm KG1. It is permanently fitted in front of the exciter filters to reduce heat.

By the use of the above lamp and filters, long wave U.V. light

(360 nm) was transmitted which was reflected from a mirror through the condenser.

The ground sections varying in thickness from 100-110 microns were examined in the darkroom at X35 and X100 magnifications. The condenser was adjusted close to the slide and the diaphragm was open to its maximum. These two settings were found to be ideal for viewing at both magnifications. The types of fluorescence and their intensities were recorded and graded subjectively. The maximum intensity was graded as 6+ and the minimum as 1+. The bluish white autofluorescence was graded as zero.

For ultraviolet photomicrography a yellow filter K430 or K460 was used in the optical path of the microscope between the specimen and the photographic film. These filters absorb or decrease the transmitted U.V. light which is stronger photographically than the fluoresced light.

Microradiographic Technique

After the ground sections were examined and microphotographed in ultraviolet and ordinary light, they were further examined by microradiographic technique. Earlier experience has shown that thin ground sections of teeth and the core of bioplastic material in which they are normally embedded tend to warp and curl on drying. The second problem encountered was that the tooth section tended to separate from the core of the plastic. Thus, the attempts commonly used to keep the sections flat and perfectly in contact with the radiographic film are not usually successful. Thirdly, when cross sections of rat incisors are used, it is further complicated because of the very small size of the tooth section. Even though the plastic core is pressed hard against the film it still does not ensure the perfect contact of the tooth section with the

film. To ensure a very close contact of the tooth section with the radiographic film, which greatly reduces the penumbral radiation scatter, a special cassette was designed and constructed which is illustrated and described in detail in Figure 8.

X-ray Equipment

A General Electric X-ray machine with an improved Coolidge x-ray tube, equipped with copper anode and tungsten target of focal spot 1.5 mm square was used as is shown in Figure 9. The unit was operated at 22.5 KV and 5 MA and a target film distance of 10 cm. The exposure time for sections of approximately 100 microns was 5 minutes. Maximum of 6.5 minutes exposure was utilized in certain instances where sections were between 100 to 110 microns thick.

With the x-ray unit operating at 22.5 KV the intensity of the hetrogenous component of the radiation is small compared to that of characteristic ka radiation of copper (1.54 A^o). Therefore the microradiographs were obtained with nearly monochromatic radiation.

Because of its high resolving power Eastman Kodak 649-0 type emulsion film was employed. Tooth sections to be studied were dried with a tissue paper, and placed in direct contact with 12 mm round piece of radiographic film in the specially designed cassette (Figure 8) in a dark room under a safe light (Kodak Wratten Series 6B). The whole assembly was then placed below the x-ray tube and subjected to x-ray exposure in a dark room.

The exposed films were processed as follows: they were first immersed under agitation, in Eastman Kodak D-19 Developer for 3.5 minutes at 70°F, and then for 30 seconds in a Kodak Acid Stop Bath, and in running water for a similar period, and finally they were exposed to a

Kodak Acid Fixer for 8 minutes. The films were then placed in Kodak Hypoclearing Agent for 3 minutes, washed in running water for 30 minutes and finally immersed in photoflow for 30 seconds. The microfilms were dried in a dust free room for at least 24 hours and then mounted in permount without dehydration and small weights were placed over the cover slips until the mounting medium was hard. This ensured flat contact of the microfilm with the glass slide and cover slip.

TABLE III

TECHNIQUE SHOWING TWO STEPS OF THREE POINT SMOOTHING OF ORIGINAL DAILY WEIGHTS OF RATS INJECTED WITH TETRACYCLINE HCl 200 mg/kg BODY WEIGHT

DAY	Weight in Grams		
	Original	1st Smoothing	2nd Smoothing
1	89	-	-
2	95	97.00	-
3	109	105.33	105.66
4	114	114.66	113.77
5	123	121.33	120.11
6	127	124.33	123.88
7	123	126.00	125.11
8	128	125.00	125.00
9	124	124.00	124.22
10	120	123.66	124.44
11	127	125.66	126.88
12	130	131.33	131.55
13	137	137.66	137.77
14	145	144.33	144.66
15	151	152.00	151.66
16	160	158.66	158.88
17	165	166.00	166.55
18	173	175.00	175.00
19	187	184.00	184.00
20	192	193.00	192.33
21	200	200.00	200.33
22	208	208.33	207.99
23	217	215.66	215.44
24	222	222.33	-
25	228	-	-

Figure 1:

The effect of two applications of 3 point smoothing to a growth curve in rats injected with 200 mg/kg of tetracycline HCl.

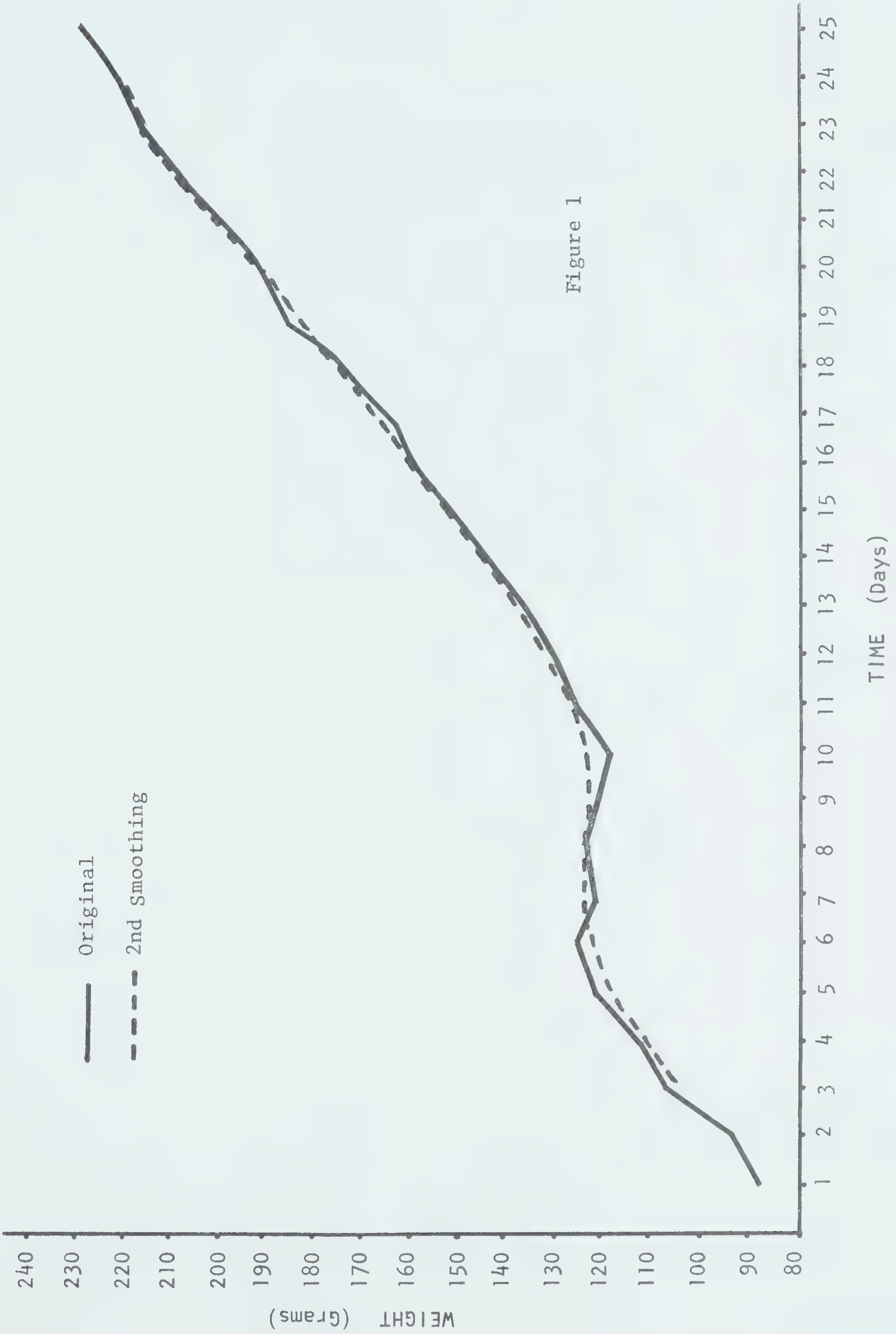

Original
2nd Smoothing
WEIGHT (Grams)
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
TIME (Days)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Figure 1

Figure 2:

Illustration of Measurements Taken from the Radiographs of Femur and Humerus

A. It is a photograph of the radiographs taken of femur and humerus. It shows quite distinct outline and density of the bones.

B. It shows tracing of the radiographs and location of reference points on femur and humerus. A line is drawn by joining point A and B. From point E a line is drawn parallel to the line AB. The distance between two lines, i.e. AB and CD is measured and expressed as length measurement.

Figure (2a)

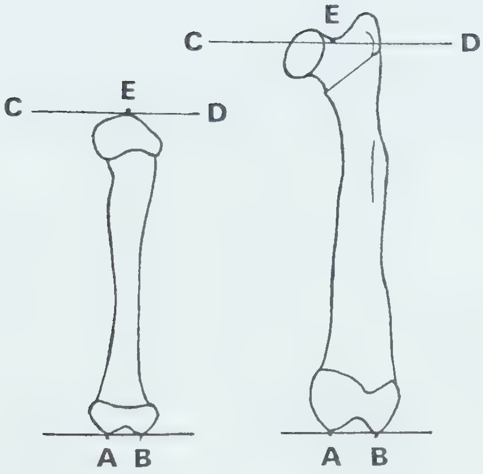


Figure (2b)

Figure 3:

Gram-O-Matic Precision Balance Adapted for Weighing Bones in Air and Water.

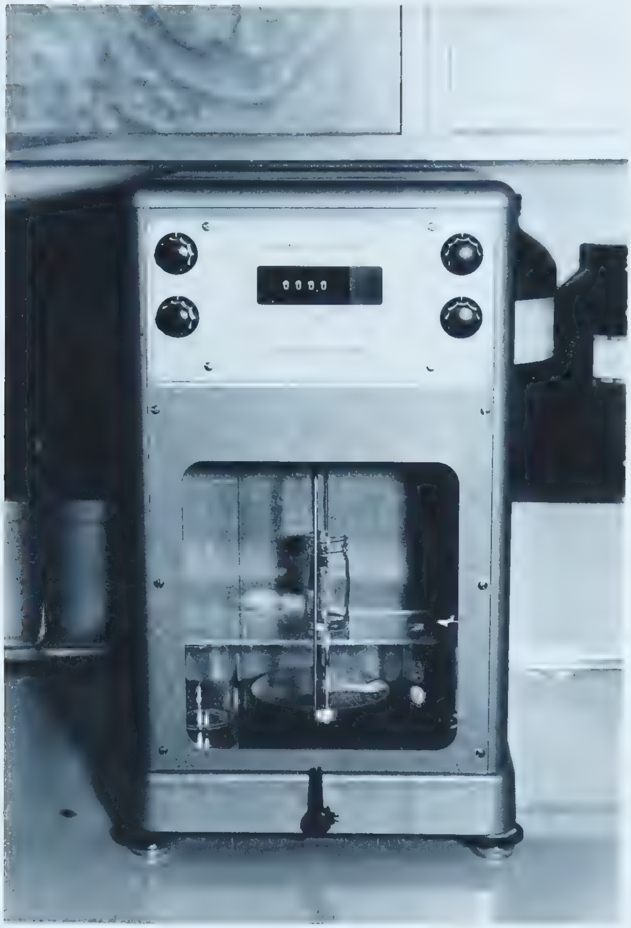


Figure 3

TABLE IV

PERCENTAGE DIFFERENCES IN REPEAT MEASUREMENTS OF FEMUR AND HUMERUS WEIGHTS; VOLUMES AND DENSITIES OF TWO GROUPS OF ANIMALS

Bone	Animal Number	Weight (grams)			Volume (Cm^3)			Density		
		Initial	After 2 weeks	% Difference	Initial	After 2 weeks	% Difference	Initial	After 2 weeks	% Difference
FEMUR	6	0.4395	0.4499	+2.37	0.3522	0.3512	-0.28	1.248	1.278	+2.40
	7	0.5545	0.5624	+1.42	0.4084	0.4024	-1.47	1.369	1.397	+2.05
	8	0.5092	0.5377	+5.60	0.3887	0.3887	±0.00	1.310	1.383	+5.57
	Mean	0.5011	0.5167	+3.11	0.3831	0.3808	-0.60	1.309	1.353	+3.36
	37	0.4855	0.4940	+1.75	0.3639	0.3645	+0.16	1.334	1.355	+1.57
	38	0.4197	0.4285	+2.10	0.3129	0.3183	+1.73	1.335	1.346	+0.82
	40	0.5020	0.5040	+0.40	0.3702	0.3699	-0.08	1.359	1.363	+0.29
	Mean	0.4691	0.4755	+1.36	0.3490	0.3509	+0.54	1.343	1.355	+0.89
HUMERUS	6	0.2013	0.2151	+6.86	0.1608	0.1604	-0.25	1.252	1.341	+7.11
	7	0.2690	0.2694	+0.15	0.1890	0.1874	-0.85	1.425	1.438	+0.91
	Mean	0.2352	0.2423	+3.02	0.1749	0.1739	-0.57	1.339	1.390	+3.74
	37	0.2288	0.2358	+3.06	0.1682	0.1697	+1.01	1.360	1.390	+2.21
	40	0.2333	0.2347	+0.60	0.1687	0.1693	+0.36	1.382	1.386	+0.29
	Mean	0.2311	0.2353	+1.82	0.1685	0.1695	+0.59	1.371	1.388	+1.23

Figure 4:

Photometric Measurement Apparatus

A. Photo-volt light meter Model 501M. Photometric scale reads from 0 to 100. The controls include a four position sensitivity range switch.

B. Search unit equipped with photo tube type C of spectral response between the range of 375-650 nm wave length is connected to the volt meter (A) with flexible twin cords.

C. M5 Wild Dissecting Microscope with objective power 0.6 and eye piece power 10, giving total magnifying power of six times.

D. Special plexiglass tray marked with a grid to hold bones in reproducable geometric positions.

E. Black-Ray ultraviolet lamp with spot bulb (340-380 nm). The radiation is incident at approximately 45° to the specimen in the tray at a distance of ten inches.

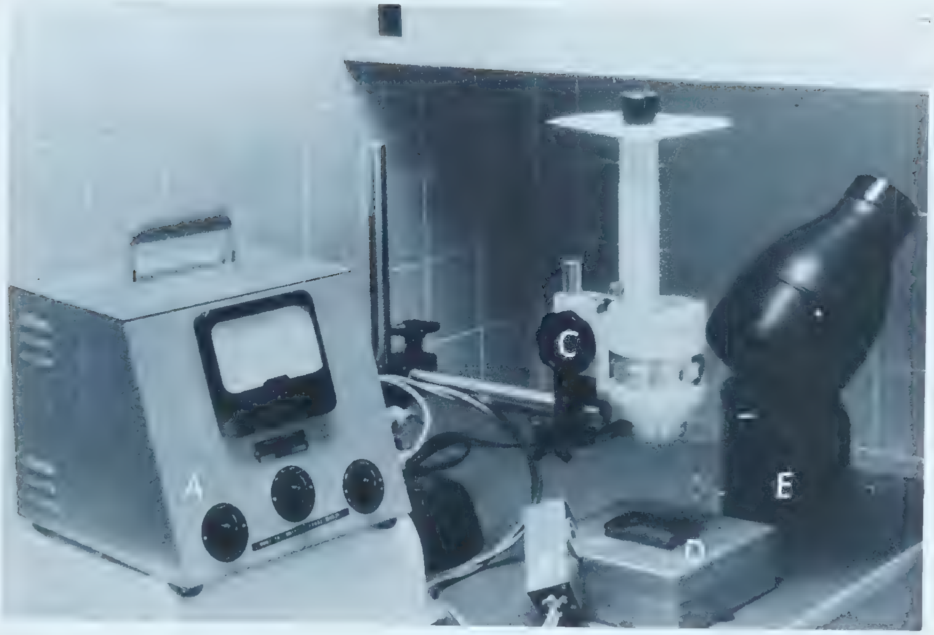


Figure 4

Photometric Measurement Apparatus

Figure 5:

Set Up for Ultraviolet Photography

A. Leitz Wetzlar stand equipped with Ernst Leitz camera; view finder; extension bellow; diaphragm (aperture 4.5 to 32) lens (Leitz Hektor f=13.5 cm, 1:4.5 and shutter release cord). Kodak Wratten filter K1 (6) in conjunction with Ansco filter No. 17 was used on top of the lens.

B. Ultraviolet lamp (Black-Ray) (340-380 nm). The radiation is incident at approximately 45° to the specimens at a distance of 10 inches.

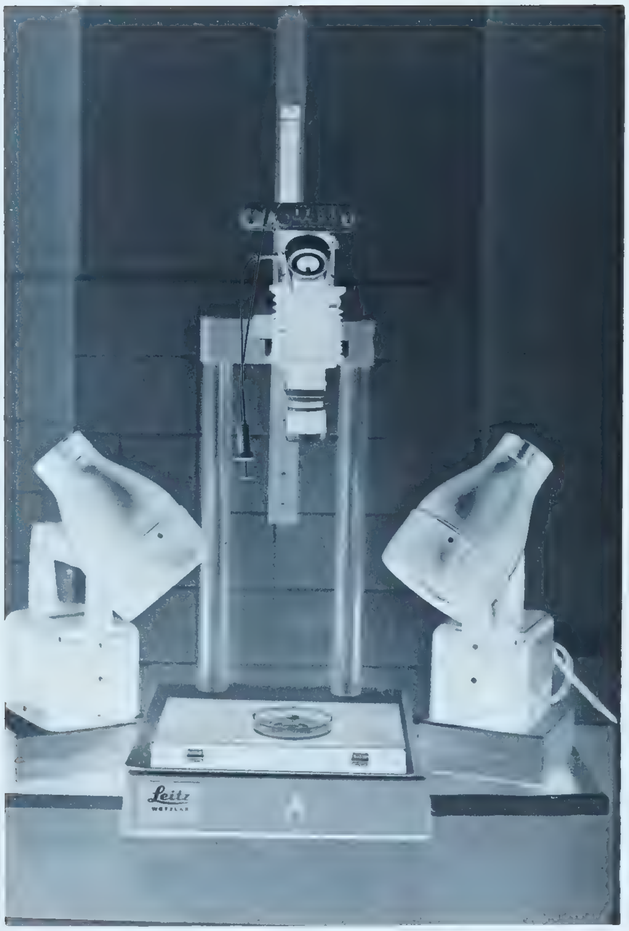


Figure 5

Set Up for Ultraviolet Photography

Figure 6:

Gillings Thin-Sectioning Machine

Lateral view shows:

A. Diamond wheel .012 inches thick (approximately 305u).

B. Bioplastic block containing specimen is attached to the working plexiglass sheet which in turn is fastened to the fixed mounting block with screws.

C. Measuring Gauge: The dial of the gauge is divided into 50 units and each unit is further subdivided in two units. One complete turn of the needle register 0.5 mm or 500 microns; thus one subunit represents 5 microns.

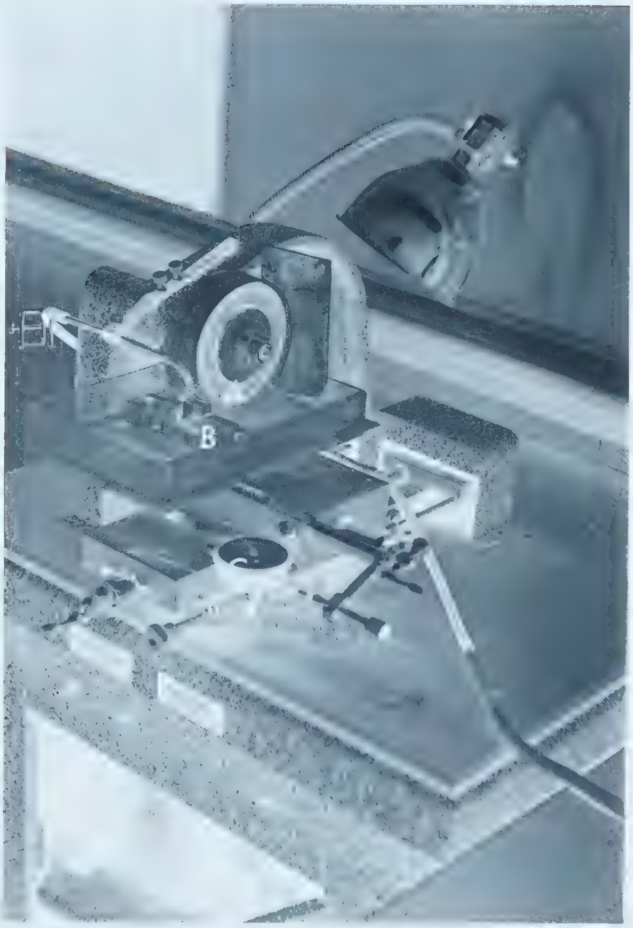


Figure 6

Gillings Thin Sectioning Machine

Figure 7:

Fluorescence Photomicrographic Equipment

A. Leitz Dialux-Pol Microscope equipped with:

- Leitz camera, view finder and shutter release cord.
- Zernike phase contrast fluorescence condenser 402 b (setting H).
- objective lenses x3.5 and x10
- occlular lens x10
- suppression filter K460 interposed between the objective lens and the occular lens. (to absorb U.V. light)

B. Mirror housing - allows the use of high pressure or low pressure lamps alternately.

C. Universal lamp housing with:

- Osram HBO 200W high pressure mercury vapor lamp.
- Heat absorption filter - 2 mm KG 1
- U.V. Excitor filter 2 mm UG 1

  2+2 mm UG 1
- Reducer of residual red transmission of excitor filter 4 mm BG 38.

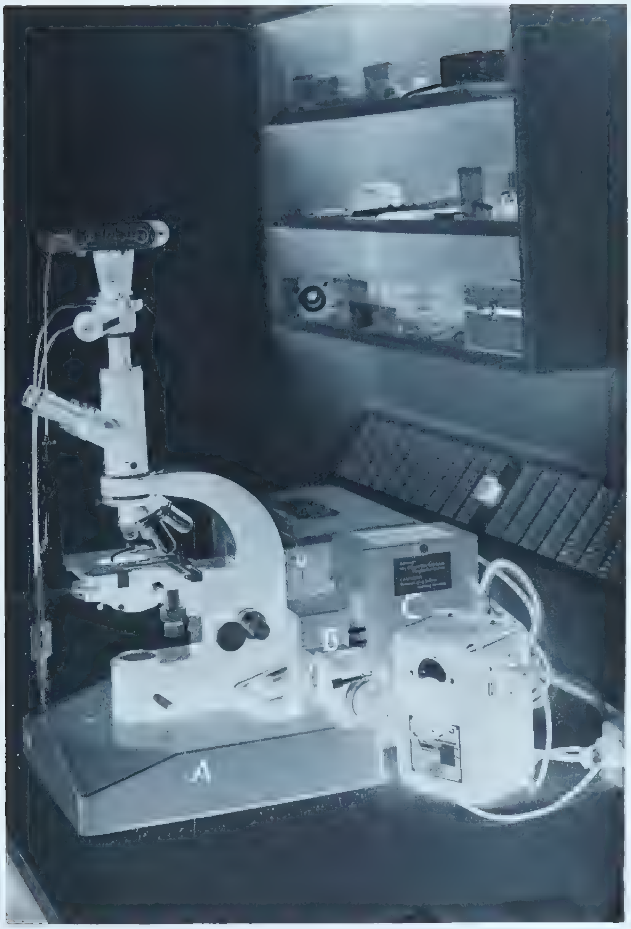


Figure 7

Fluorescence Photomicrographic Equipment

Figure 8:

Section Holder for Microradiography

A. Thick plastic plate with a recessed area 2.5 mm in depth of the size of Kodak High Speed Microradiographic Glass Plates. The bottom of the recess is lined with black rubber. Six bolts project upwards.

B. Kodak High Speed Microradiographic Glass Plate.

C. Rubber plate with two round holes.

D. Plastic plate with six holes to fit on top of Plate A. There are two large bevelled holes in the plate through which sections are irradiated. Rubber plate (C) is temporarily cemented to the plastic plate (D). (see Methods)

E. Wing nut.

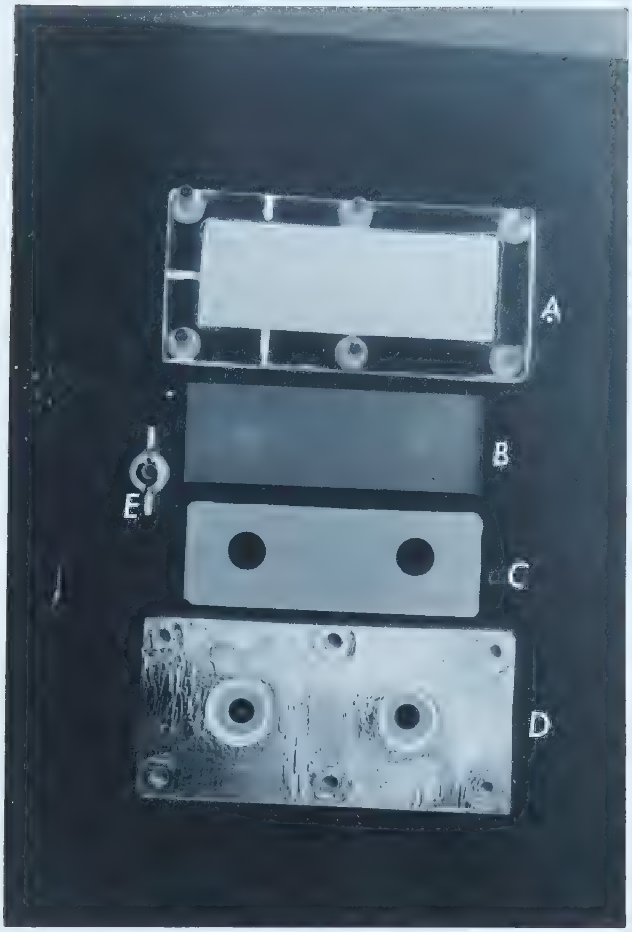


Figure 8

Section Holder for Microradiography

Figure 9:

Radiographic Unit for Contact Microradiography

A. A portable control unit showing volt meter (KV can be adjusted between range of 10-40); MA scale (at top of the unit; range between 2-5) and electronic automatic shut off timer (maximum 5 minutes).

B. Improved Coolidge X-ray Tube; equipped with copper anode and tungsten target of focal spot 1.5 mm square. The tube port contains an additional removable lead diaphragm with 1.5 cm circular central aperture.

C. Specially designed section holder in place at a distance of 10 cm below the tube port.

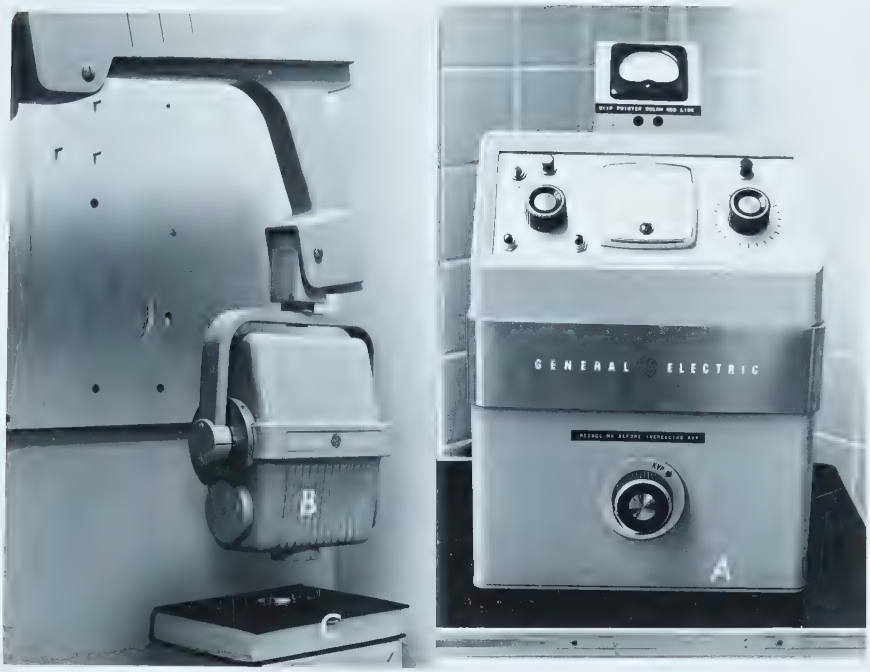


Figure 9

Radiographic Unit for Contact Microradiography

RESULTS

The experimental results of the effects of four tetracycline drugs are presented according to the following scheme.

A. Effects of the tetracyclines on growth, as measured by total body weight. (Tables V to IX inclusive and Figures 10 to 22 inclusive).

B. Effects of tetracyclines on bone.
  - (1) Physical measurements
    - (a) Lengths and weights of femora and humeri (Tables X and XI)
    - (b) Volumes and densities of femora and humeri (Tables XII and XIII)
  - (2) Mineral content of bones
    - (a) Relative weights of organic and inorganic constituents of femora and humeri (Tables XIV to XVII inclusive).

C. Discoloration and fluorescent color intensity of bones and teeth.
  - (1) Type of discoloration and fluorescent color intensity of femora and humeri.
    - (a) Gross assessment (Table XVIII)
    - (b) Photometric measurements (Table XIX, Figure 23)
  - (2) Type of discoloration and degree of fluorescent color intensity of mandibular and maxillary bones and teeth.
    - (a) Gross assessment (Tables XX and XXI)
    - (b) Photographic records (Figures 24 to 35 inclusive)
  - (3) Comparisons of fluorescent color intensities of tibiae (Figures 36 to 39 inclusive).
  - (4) Histological examination and evaluation of fluorescent color intensities in undecalcified cross sections of incisor teeth.

(a) Subjective evaluation (Table XXII and XXIII)

(b) Photographic records (Figures 41 to 49 inclusive).

D. Microradiographic observations of undecalcified cross sections of incisor teeth.

(a) Microradiographs (Figures 50 and 51)

(b) Interpretation of Microradiographs (Table XXIV)

A. Effects of the tetracycline drugs on growth

The effects of TC, OTC, CTC, and DCTC at four different dose levels are presented in Tables V through IX. These same data are represented, graphically, in figures 10 to 22. It is evident that at the 10 mg/kg dose level, growth was not retarded. In fact, TC and OTC appear to have a stimulatory effect, as is indicated by the growth curves lying at the upper limit of the range of the Control values. However, as the doses were increased, it is evident that growth was retarded, but at different rates by the different drugs.

At the 40 mg/kg dose levels, (Figure 16) the growth curves for all the drugs lie well within the normal range except for CTC where the curve was lying close to the lower range of the Control animals.

Figure 17 shows the effect of 80 mg/kg dose level on the growth of the animals. The inhibition in growth by CTC was quite marked in comparison to the effects of the other three drugs. During the period when CTC was injected, i.e. between the 5th and 11th day, no increase in weight was observed. But as the drug was withdrawn growth appeared to continue normally and came to lie within the range of control group between the 17th and 18th day. OTC and DCTC also caused some inhibition in growth during the time the drug was administered as the curves lie outside the lower range of the control group.

Figure 18 shows the comparative effect of 4 tetracycline drugs at 200 mg/kg dose level. All the drugs affected growth to a varying extent. The minimum effect was observed with DCTC and the maximum effect was observed with CTC. The effect of CTC was so severe that three animals died during the first three days of drug administration. OTC not only caused cessation but also marked loss of body weight. There was a continued decrease in body weight during drug administration periods and this trend continued for 1-2 days after the drug was withdrawn. In subsequent days, there was a marked increase in the rate of growth as is evident by the upward slope of the curve which then becomes parallel to the control curve. TC caused complete cessation of growth during 6 days of injection period but growth resumed later in a normal manner. This effect was less marked in relation to CTC and OTC. However, none of the body weights of any of the animals lay within the range of the control group during the whole experimental period.

Figures 19, 20, 21 and 22 offer a comparison of the effects of the drugs when used on a weight to weight basis. DCTC showed consistently a non-inhibitory effect on growth at all dose levels than did the other three drugs. (The therapeutic dose of DCTC is usually one fourth the dose of the other three drugs.)

B. Effects of tetracyclines on bone

(1) Physical Measurements

Table X and XI record the length and weight measurements of rat femora and humeri at the termination of the experiments. The bones show normal increase in length at the 10 mg/kg dose level with all the four drugs. OTC and CTC appear to have caused some reduction in length at

200 mg/kg dose level, whereas DCTC appears to have had no effect. A definite increase in weight of bones is observed at 10 mg dose level. In equivalent amounts DCTC appeared to have no adverse effect on the weight of the bones at all four dose levels tested. TC caused the greatest deficiency in bone weight at the 200 mg/kg dose level in comparison with the other drugs, except for DCTC where an actual increase in the weight of the bones was observed.

Table XII shows an increase in volume of the bones following TC at the 10 mg/kg dose level. It is interesting to note that there is some indication that at the 40 mg/kg dose level the volume appears to be somewhat deficient and at 80 mg/kg dose level, it was larger than the normal controls. Except for DCTC, there was a definite deficiency in volume at the 200 mg/kg dose level. OTC appears to have effected the greatest deficiency in bone volume, whereas DCTC had no effect at comparable dose levels.

Table XIII presents the calculated densities of the bones and shows that the bone densities in the TC drug group are lower than the corresponding controls and the bone density decreases as the dose of the drug was increased, i.e. the bone density of 1.264 at 10 mg/kg dose level (control 1.303) has continuously decreased to 1.196 at the 200 mg/kg dose level. It appears that the other three drugs have caused no appreciable difference in density at all four dose levels tested. In fact, the density has increased in comparison to the control group.

(2) Mineral content of bones

Table XIV, XV, XVI, and XVII record the weights of the organic constituents of bones and the calculation of the portion (percent) of the inorganic content. The difference between the weights before and after

decalcification was taken as the inorganic constituents of the bones. Comparison of these tables show that at all dose levels there was a decrease in the inorganic fraction of the bone. At the 10 mg/kg dose level 5.56% decrease was observed following CTC, whereas TC and DCTC resulted in a reduction of approximately 2% of the inorganic fraction. Least decrease (1.4%) was observed following OTC. As the dose was increased to 200 mg/kg the maximum decrease of 13.35% was noticed following administration of TC. OTC resulted in decrease of 8.45% whereas CTC and DCTC both caused decreases of approximately 5%. The above findings are quite consistent with the results obtained for length, weight, volume and density of bones of animals injected with TC. At the 200 mg/kg dose level, administration of TC resulted in a definite decrease in weight, volume, density and inorganic constituents of bones and this effect appears to be less marked with the other three drugs. DCTC is seen to effect bone the least with respect to reduced weight, volume and density.

C. Discoloration and fluorescent color intensity of bones and teeth

Three methods were applied in this study to assess the type and amount of colored fluorescence displayed by bones and teeth.

(1) Gross assessment (subjective evaluation)

(2) Photometric measurement

(3) Histological examination of ground sections of teeth

(1) Gross assessment

Gross examination of both femora and humeri of animals showed no visible anatomical defects or abnormalities. In comparison with the control group, all the bones showed varying degrees of discoloration and fluorescence which was dependent upon the dosage and the type of tetra-

cycline drug used. OTC caused the least discoloration (light grey) whereas TC was found to cause the maximum discoloration (brownish yellow) (Table XVIII).

In order that some subjective interpretation of the discoloration and fluorescence given by these drugs be made, the bones were arranged in descending order from maximum fluorescence to the lowest fluorescence and scores were made ranging between 10+ to 1+. The scores for the teeth were made ranging between 7+ and 1+ because the range of intensity of coloration was narrower than in the case of bones.

The findings of such an analysis are presented in Tables XVIII, XIX and XX and figures 24 through 39.

Table XVIII records the fluorescent color intensity of the femora and humeri of the experimental animals. The least fluorescence was observed in those given OTC and the maximum in those which had received TC at comparable dose levels. At the normal dose level, i.e. 40 mg/kg body weight, both TC and DCTC caused the same type and amount of fluorescence (medium yellow 5+) under ultraviolet light. TC at the 80 mg/kg dose level gave fluorescence (yellow 9+) almost equal to the fluorescence given by DCTC (deep orange yellow 9+) and CTC (yellow 8+) at 200 mg/kg dose level.

(2) Photometric measurements

The above findings were further varified, semiquantitatively, by a photometric method, the results of which are presented in Table XIX and figure 23. The bar graph shows the fluorescent color measurements made on femora and humeri. The light emitted by bones in the control group was found to be slightly higher than 100%, i.e. 2-3% more, since no mechanism was available on the voltmeter to adjust the light of control to 100, the measurement of other bones were made between the range of

0-100 as was indicated on the dial of the voltmeter. As the fluorescence increased from whitish-blue (OTC, TC at 10 mg/kg) through medium yellow (TC, DCTC at 40 mg/kg) to deep orange-yellow (TC and DCTC at 200 mg/kg), the percent emitted light recorded by the photocell decreased. At each comparable dose level, TC was found to have the maximum fluorescence compared to the other three drugs. The fluorescence continued to increase progressively from 10 mg/kg to 200 mg/kg in all the four tetracycline drug groups. A small difference in fluorescence was observed between CTC and DCTC at equivalent dose levels.

Table XX and XXI show the discoloration and fluorescent color intensity of maxillary and mandibular bones and teeth respectively. They further compare the effect of sunlight on the discoloration and fluorescence given by the bones and teeth.

Under ordinary light the changes ranging from the light grey (OTC, CTC at 10 mg/kg) to light brown (OTC at 40 mg/kg) to deep brown yellow (TC, DCTC at 200 mg/kg) were seen. At any given dose level, the order of discoloration was $\mathrm{TC}=\mathrm{DCTC}>\mathrm{CTC}\gg\mathrm{OTC}$. Exposure to sunlight appears to have increased the discoloration slightly for all the drug groups except that of OTC which showed no change. All the bones and teeth tended to turn brown following exposure to sunlight.

Under ultraviolet light, bones and teeth showed a brilliant yellow fluorescence in all the drug groups except OTC. The degree of observed fluorescence was directly dependent upon the doses of the drug administered (Figures 24 to 31). At any given dose level the intensity of fluorescence was $\mathrm{TC}=\mathrm{DCTC}>\mathrm{CTC}\gg\mathrm{OTC}$ (Figures 32 to 35). At the 10 mg/kg dose level, the fluorescence was whitish-blue (Figure 32), which changed to deep orange yellow at 200 mg/kg (Figure 35). The drugs at 10 and 40 mg/kg

dose level resulted in little difference in fluorescent color intensity. The exposure to sunlight appears to have decreased the fluorescence in the OTC group, whereas in the same circumstance some increase in fluorescence was observed with the other three drugs.

With respect to the long bones (femora, humeri and tibiae) the central area of the shaft did not demonstrate as much fluorescence as the epiphyses. The midsection may have been largely calcified prior to drug administration. The fluorescence was also observed in the articular surfaces of the long bones and did not appear to be due to tetracycline deposition in the uncalcified hayline cartilage. It probably can be attributed to the fluorophore depositing in the underlying bony spicules and fluorescing through the cartilage. The distribution of fluorescence and the comparative fluorescent color intensity exhibited by the tetracycline at 10, 40, 80 and 200 mg/kg dose level is presented in Figures 36 to 39 and Table XVIII which has already been discussed.

(3) Histological examination of ground sections of teeth

Tables XXII and XXIII and Figures 40 through 49 present the evaluation of discoloration and fluorescence in ground cross sections of incisor teeth. Ground sections were examined under the microscope in ordinary and under ultraviolet light and interpretations in respect of type of coloration and intensity of fluorescence were made. The detailed results were recorded in the above tables. In spite of the fact that exhaustive preliminary experiments were made to standardize the photographic technique, the colors seen by the eye could not be reproduced in making photographs of the sections. None the less, the photographs do present a meaningful comparison of fluorescent color intensities of cross section of teeth.

Examination of sections (Table XXII) under ordinary light showed

yellow markings present in the dentine except in OTC specimens where no markings were seen. It was further observed that these markings were distinct at the 10 and 40 mg/kg dose levels and as the dosage was increased to 200 mg/kg they became diffuse and indistinct and the total area occupied by these markings appeared to have become wider, relative to the area observed at the 10 and 40 mg/kg dosages. DCTC at 20 and 50 mg/kg dose levels (equivalent dose of 80 and 200 mg/kg respectively for the other drugs) resulted in distinguishable, diffuse linear markings. The areas between the lines and pulpal to the last line were also diffusely stained.

Table XXIII presents the detailed analysis of ultraviolet fluorescence observed in ground sections of teeth.

ENAMEL: No fluorescence was observed at the 10 and 40 mg/kg dose level. CTC at 80 mg/kg and DCTC at 20 mg/kg (equivalent doses) gave slight whitish-yellow fluorescence to the enamel. All the four tetracycline drugs exhibited a definite whitish-yellow fluorescence in the enamel at 200 mg/kg dose level.

DENTINE: Figures 41, 42, 43 and 44 present the fluorescence given by tetracyclines at four different dose levels. The intensity of fluorescence seen in the dentine was directly related to the dosage administered. It varied from light-yellow at low dosage to deep orange-yellow at high dose levels. Figures 46, 47, 48 and 49 show that intensity of fluorescence was $\mathrm{TC} = \mathrm{DCTC} > \mathrm{CTC} \gg \mathrm{OTC}$. All the drugs caused clear and very distinct fluorescent lines at 10, 40 and 80 mg/kg dose level. TC and OTC exhibited diffuse staining of the drug between the lines at the 80 mg/kg dose level. Furthermore, TC was found to be the only drug to stain the dentine dif-

fusely, pulpal to the last line. At the 200 mg/kg dose level with all the drugs dentine showed poorly distinguishable fluorescent lines. It appeared that drug was present between the lines and appeared as one wide band of fluorescent material. TC appears to have reduced the width of the dentine formed during the time the drug was administered. Except for the CTC group all the three drugs have stained the dentine which was formed after the last dosage of the drug was given. DCTC at 50 mg/kg (equivalent of 200 mg/kg for others) has stained the dentine deep orange-yellow color, pulpal to the last line.

D. Microradiographic Studies

Microradiographs, when compared with photomicrographs of the same ground sections, reveal that radiolucent bands are present which correspond to the bands of tetracycline discoloration.

TABLE V

AVERAGE DAILY WEIGHTS OF RATS INJECTED WITH PHYSIOLOGIC SALINE (CONTROL GROUP)

DAY	Weight in Grams		
	Original	1st Smoothing	2nd Smoothing
1	90	-	-
2	96	97.00	-
3	105	105.00	105.22
4	114	113.66	113.33
5	122	121.33	121.11
6	128	128.33	128.11
7	135	134.66	134.66
8	141	141.00	141.22
9	147	148.00	148.11
10	156	155.33	155.22
11	163	162.33	162.33
12	168	169.33	168.99
13	177	175.33	175.66
14	181	182.33	182.22
15	189	189.00	187.99
16	197	192.66	192.88
17	192	197.00	197.44
18	202	202.66	203.66
19	214	211.33	210.33
20	218	217.00	218.77
21	229	228.00	227.00
22	237	236.00	235.00
23	242	241.00	240.33
24	244	244.00	-
25	246	-	-

TABLE VI

AVERAGE DAILY WEIGHTS OF RATS INJECTED WITH TETRACYCLINE HCl FOR SIX DAYS

DAYS	DOSE mg/kg BODY WEIGHT			
	10	40	80	200
1	90*	90	88	89
2	96	98	97	97
3	104	107	107	106
4	112	115	115	114
5 **	120	121	123	120
6	128	127	128	124
7	136	133	132	125
8	143	138	136	125
9	150	142	139	124
10***	158	147	143	124
11	165	152	148	127
12	172	159	154	132
13	178	166	161	138
14	185	173	169	145
15	191	179	177	152
16	197	185	183	159
17	201	192	188	167
18	206	200	194	175
19	213	208	203	184
20	222	216	213	192
21	229	223	222	200
22	236	230	230	208
23	243	236	239	215
24	249	241	246	222
25	256	246	253	228

* Grams
** First injection
*** Sixth injection

TABLE VII

AVERAGE DAILY WEIGHTS OF RATS INJECTED WITH OXYTETRACYCLINE HCl FOR SIX DAYS

DAYS	DOSE mg/kg BODY WEIGHT			
	10	40	80	200
1	90*	88	90	89
2	97	96	99	99
3	105	104	108	108
4	113	112	115	114
5**	120	119	121	115
6	127	124	126	112
7	133	129	130	106
8	141	134	134	102
9	148	138	137	98
10***	155	143	140	95
11	161	148	144	92
12	167	154	149	93
13	173	160	156	96
14	181	168	163	103
15	188	175	171	111
16	195	183	177	119
17	202	190	185	127
18	208	196	192	136
19	214	203	200	146
20	220	209	207	156
21	227	217	214	166
22	233	224	221	175
23	238	230	228	184
24	241	236	235	192
25	242	241	242	200

* Grams
** First injection
*** Sixth injection

TABLE VIII

AVERAGE DAILY WEIGHTS OF RATS INJECTED WITH CHLORTETRACYCLINE HCl FOR SIX DAYS

DAYS	DOSE mg/kg BODY WEIGHT			
	10	40	80	200†
1	89*	90	90	89
2	97	97	98	98
3	105	105	105	106
4	113	112	112	111
5 **	121	119	117	115
6	127	124	119	115
7	133	126	120	113
8	138	129	120	
9	144	132	121	
10 ***	150	136	123	
11	157	141	126	
12	163	147	132	
13	170	154	138	
14	177	162	146	
15	185	169	154	
16	192	175	162	
17	198	180	170	
18	204	186	177	
19	207	192	185	
20	212	198	193	
21	217	205	201	
22	222	213	209	
23	227	220	215	
24	231	227	221	
25	237	234	227	

* Grams

** First injection

*** Sixth injection

† After 7 days 3 animals died.

TABLE IX

AVERAGE DAILY WEIGHTS OF RATS INJECTED WITH DEMETHYLCHLORTETRACYCLINE HCl FOR SIX DAYS

DAYS	DOSE mg/kg BODY WEIGHT				
	2.5	10	20	50†	80
1	88*	88	88	92	91
2	95	96	96	99	99
3	102	103	104	107	108
4	109	111	112	115	115
5**	116	119	118	120	120
6	122	127	124	123	122
7	129	133	128	125	122
8	135	137	130	127	122
9	140	141	132	130	121
10***	146	145	135	134	121
11	151	150	138	139	123
12	157	156	143	144	127
13	163	162	147	150	132
14	170	169	152	156	138
15	176	177	158	162	144
16	183	184	164	168	151
17	190	191	171	175	158
18	198	198	177	182	165
19	205	205	185	189	173
20	212	213	192	197	180
21	218	219	199	205	189
22	225	225	205	214	197
23	231	231	210	222	206
24	237	238	216	229	215
25	243	247	224	237	224

* Grams
** First injection
*** Sixth injection
† Averages are based on weights of 3 animals in this group only.

Figure 10:

Growth Curve of Control Animals with Upper and Lower Limits of the Range.

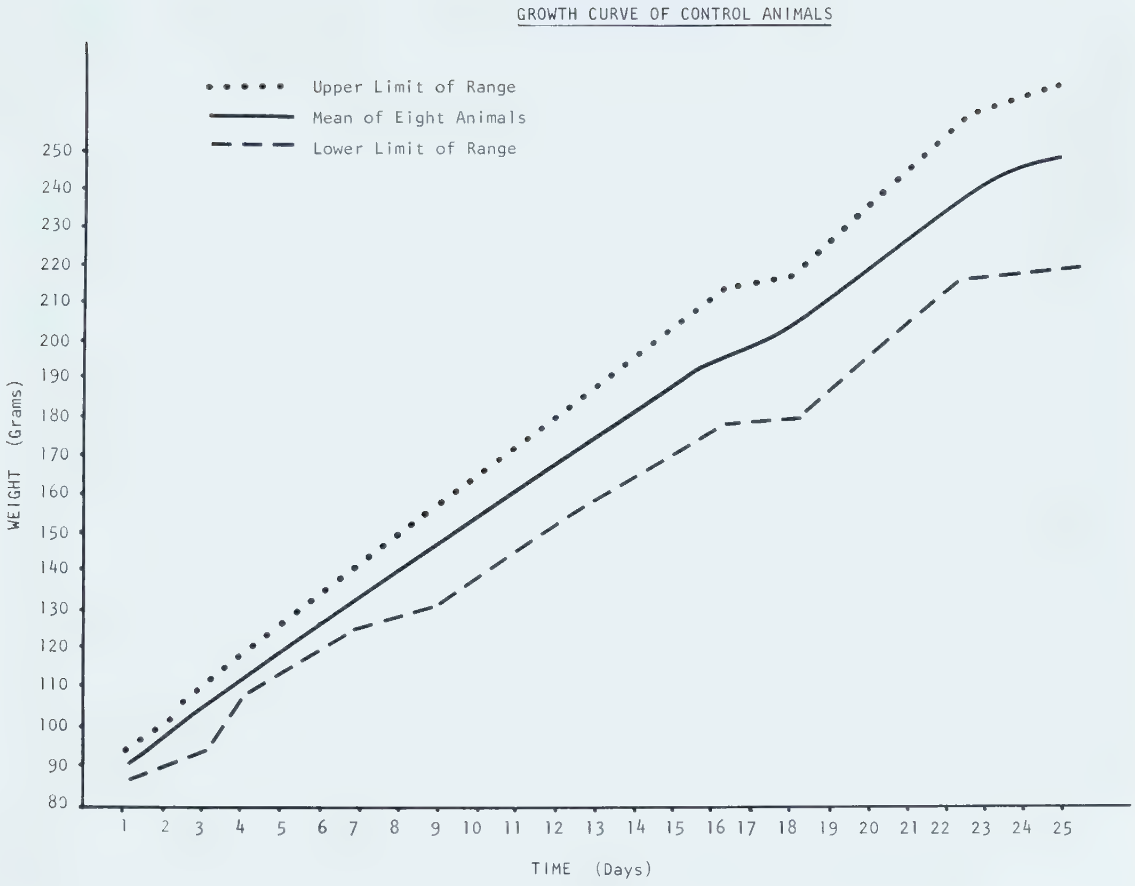
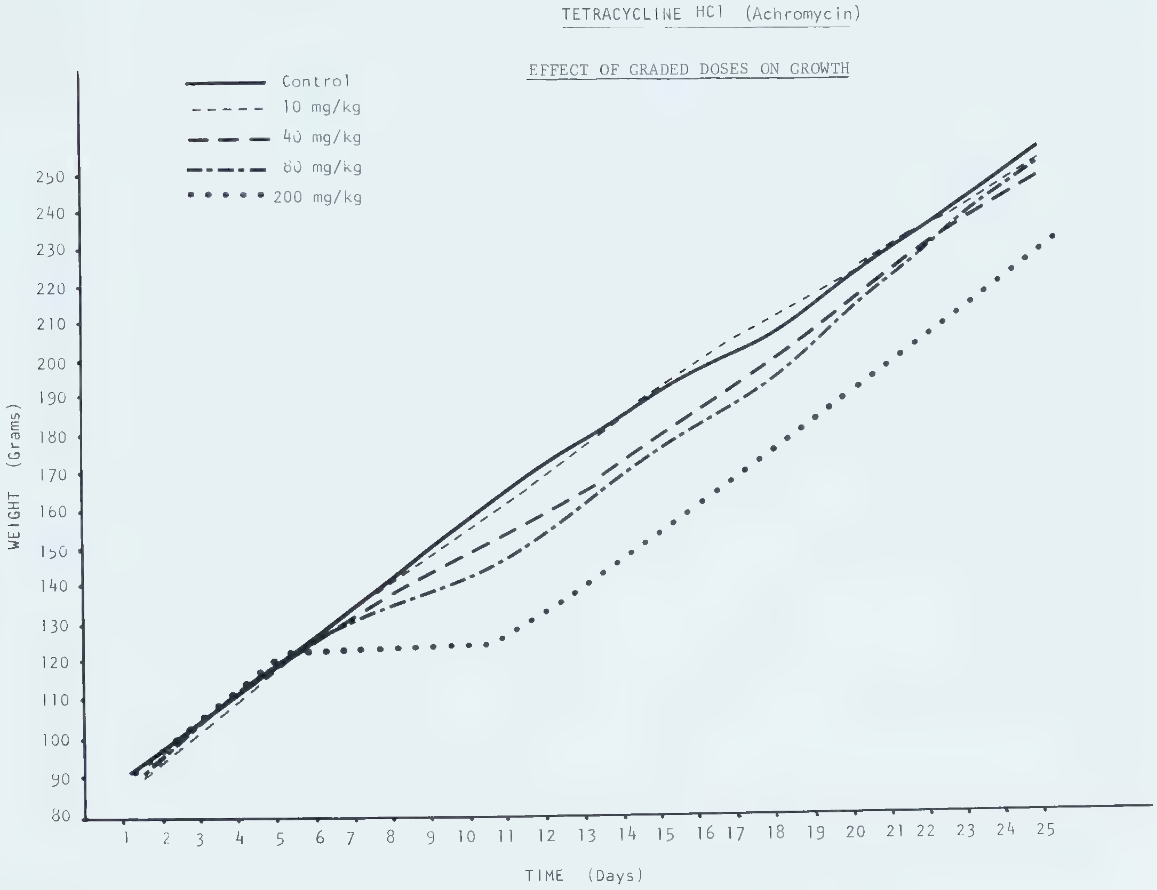

GROWTH CURVE OF CONTROL ANIMALS
• • • • • Upper Limit of Range
Mean of Eight Animals
Lower Limit of Range
WEIGHT (Grams)
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
TIME (Days)

Figure 10

Figure 11:

Shows comparison of growth curves of rats injected with tetracycline HCl (TC) at 10, 40, 80 and 200 mg dose level. Total of six injections, the first on the 5th and sixth on the 10th day of the experimental period were given. Solid line indicates untreated controls.



TETRACYCLINE HCl (Achromycin)

EFFECT OF GRADED DOSES ON GROWTH

Figure 11

Figure 12:

Shows comparison of growth curves of rats injected with oxytetracycline HCl (OTC) at 10, 40, 80 and 200 mg dose level. Total of six injections, the first on the 5th and sixth on the 10th day of experimental period were given. Solid line indicates untreated controls.

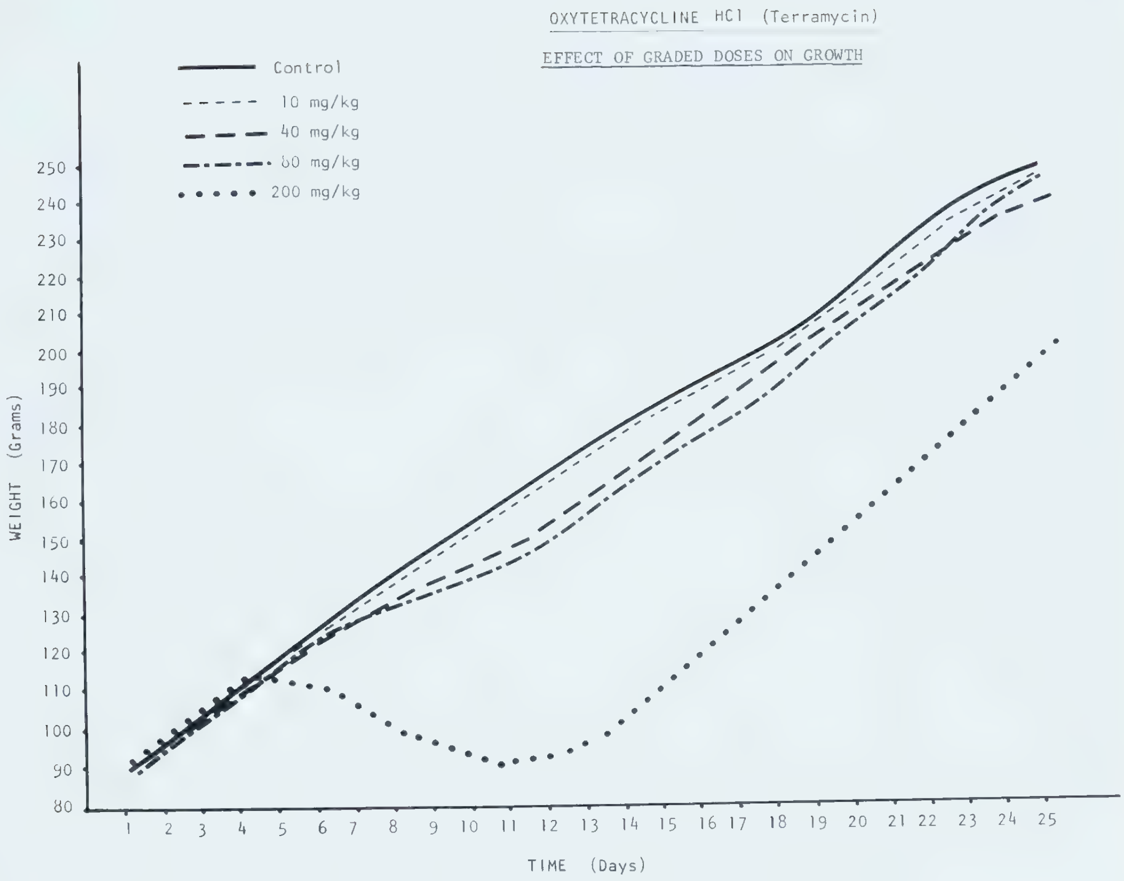

OXYTETRACYCLINE HCl (Terramycin)
EFFECT OF GRADED DOSES ON GROWTH
Control
10 mg/kg
40 mg/kg
60 mg/kg
200 mg/kg
WEIGHT (Grams)
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
TIME (Days)

Figure 12

Figure 13:

Shows comparison of growth curves of rats injected with chlortetracycline HCl (CTC) at 10, 40, 80 and 200 mg dose level. Total of six injections, the first on the 5th and sixth on the 10th day of the experimental period were given. Solid line indicates untreated controls.

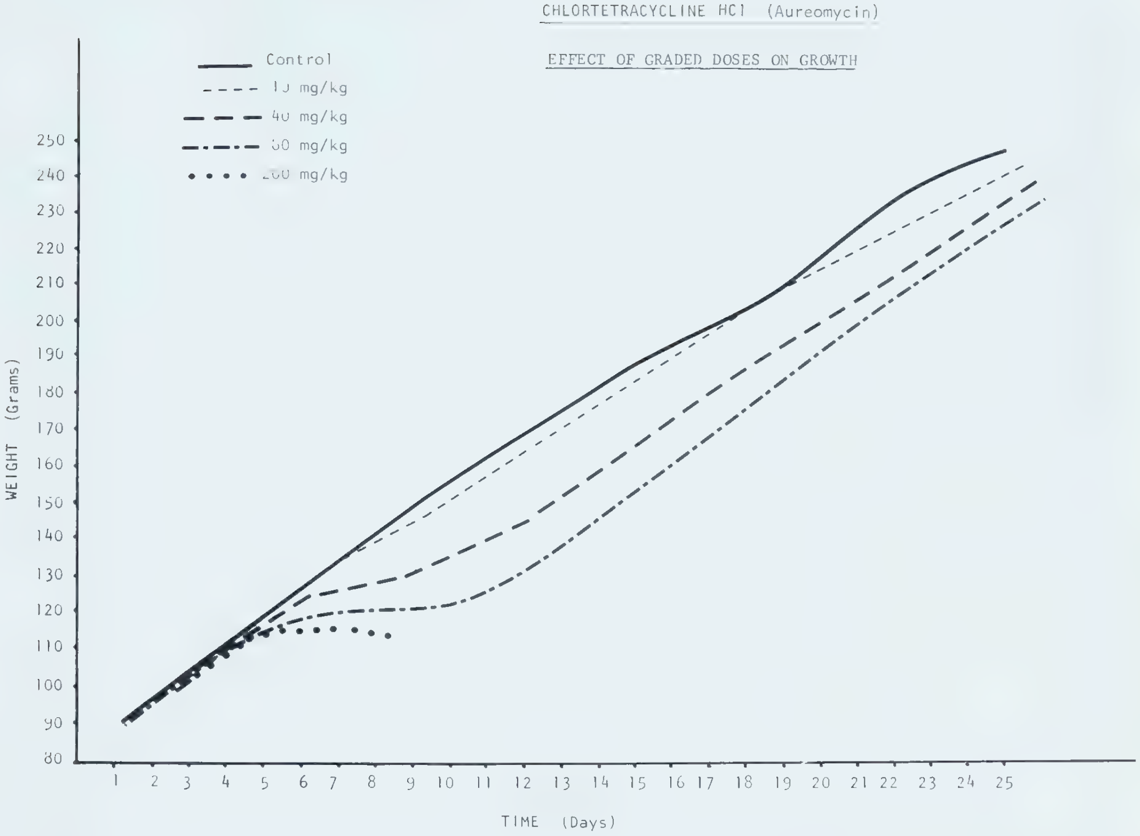

CHLORTETRACYCLINE HCl (Aureomycin)
EFFECT OF GRADED DOSES ON GROWTH
Control
10 mg/kg
40 mg/kg
60 mg/kg
200 mg/kg
WEIGHT (Grams)
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
TIME (Days)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Figure 13

Figure 14:

Shows comparison of growth curves of rats injected with demethylchlortetracycline HCl (DCTC) at 2.5, 10, 20, and 50 mg dose level. Total of six injections, the first on the 5th and sixth on the 10th day of the experimental period were given. Solid line indicates the untreated controls.

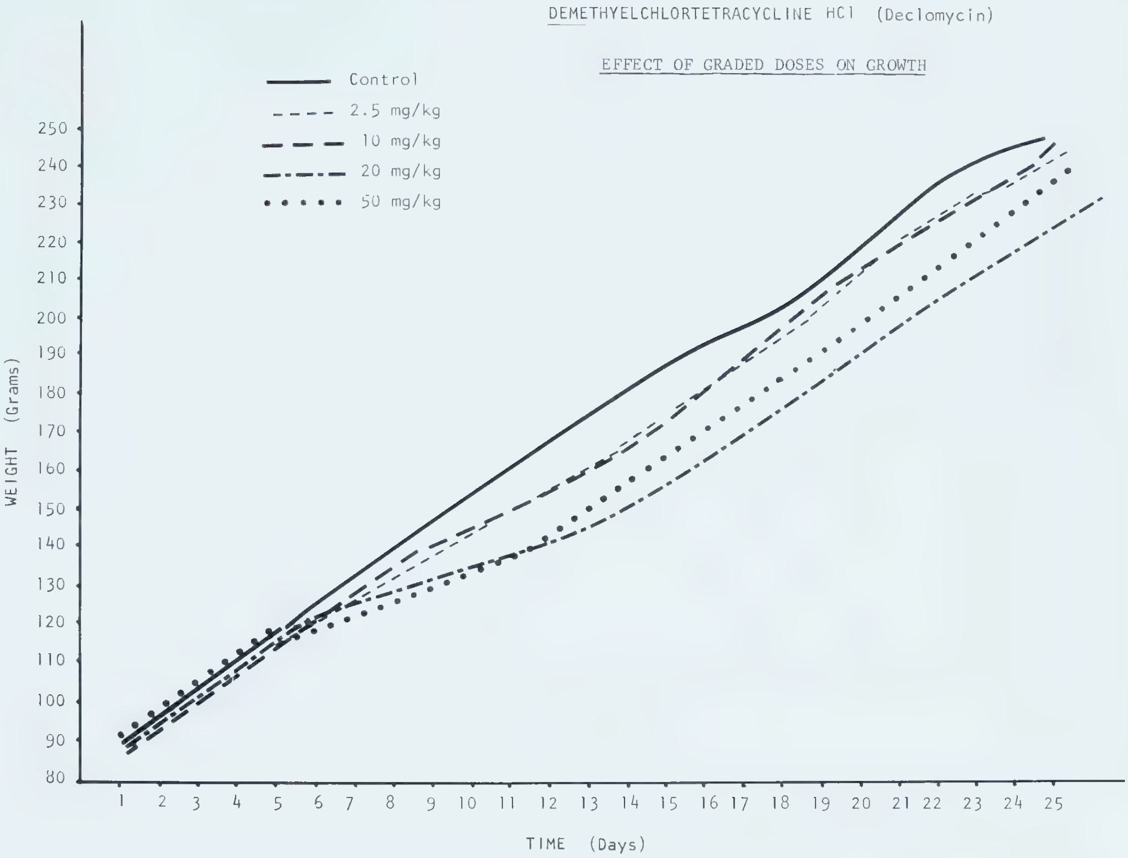

DEMETHYELCHLORTETRACYCLINE HCl (Declomycin)
EFFECT OF GRADED DOSES ON GROWTH
Control
2.5 mg/kg
10 mg/kg
20 mg/kg
50 mg/kg
WEIGHT (Grams)
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
TIME (Days)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Figure 14

Figure 15:

Shows comparison of growth curves of rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in equivalent doses. (Dose given was 10 mg/kg body weight except for DCTC where the dose was 2.5 mg/kg body weight.) Total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period were given. Solid line indicates the untreated controls.

EFFECT OF TETRACYCLINE DRUGS ON GROW

Dose = 10 mg/kg body wei

(Equivalent dose of DMCT = 2.[illegible] mg/

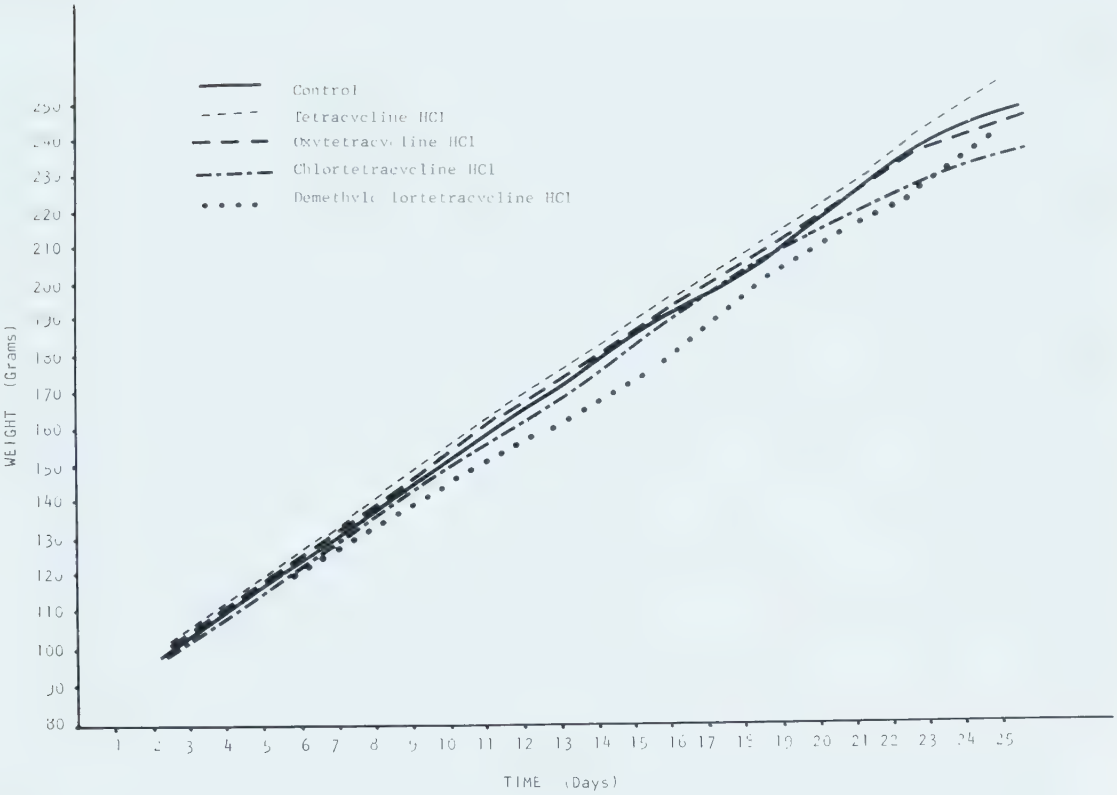


Figure 15

Figure 16:

Shows comparison of growth curves of rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in equivalent doses. (Dose given was 40 mg/kg body weight except for DCTC where the dose was 10 mg/kg body weight.) Total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period were given. Solid line indicates the untreated controls.

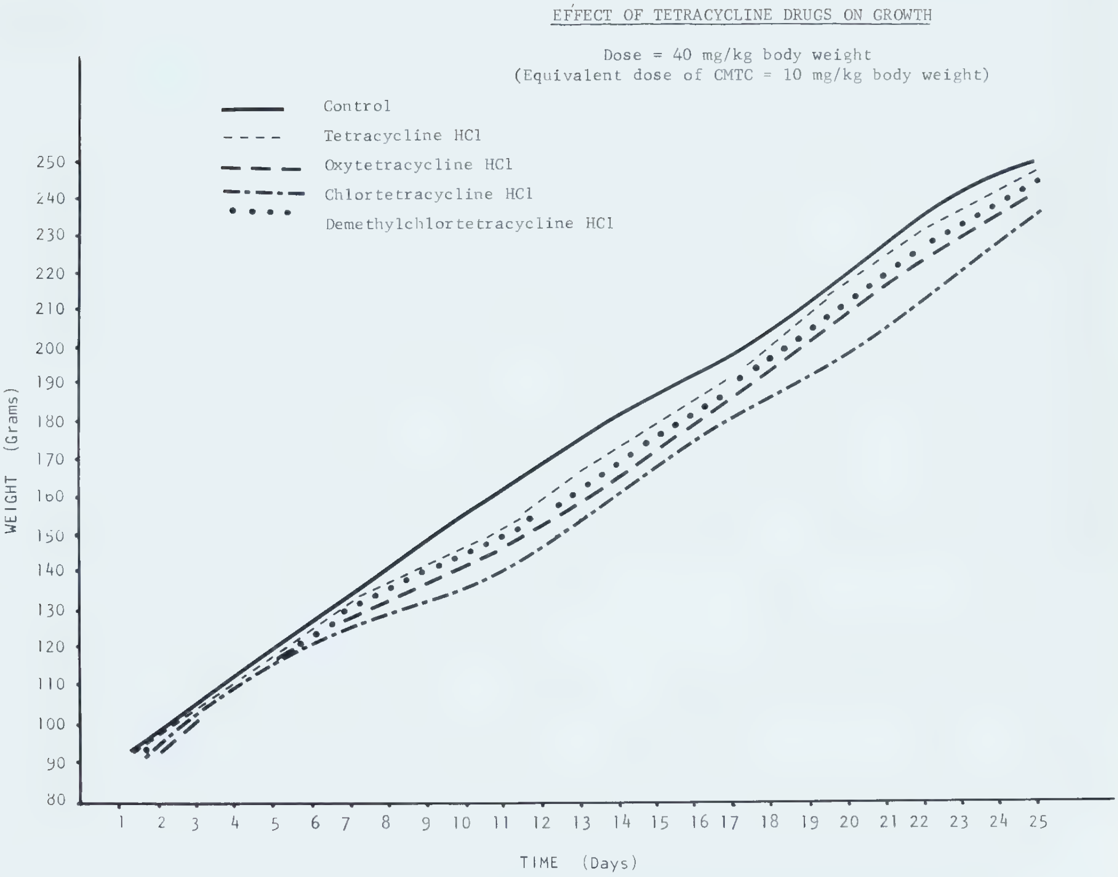
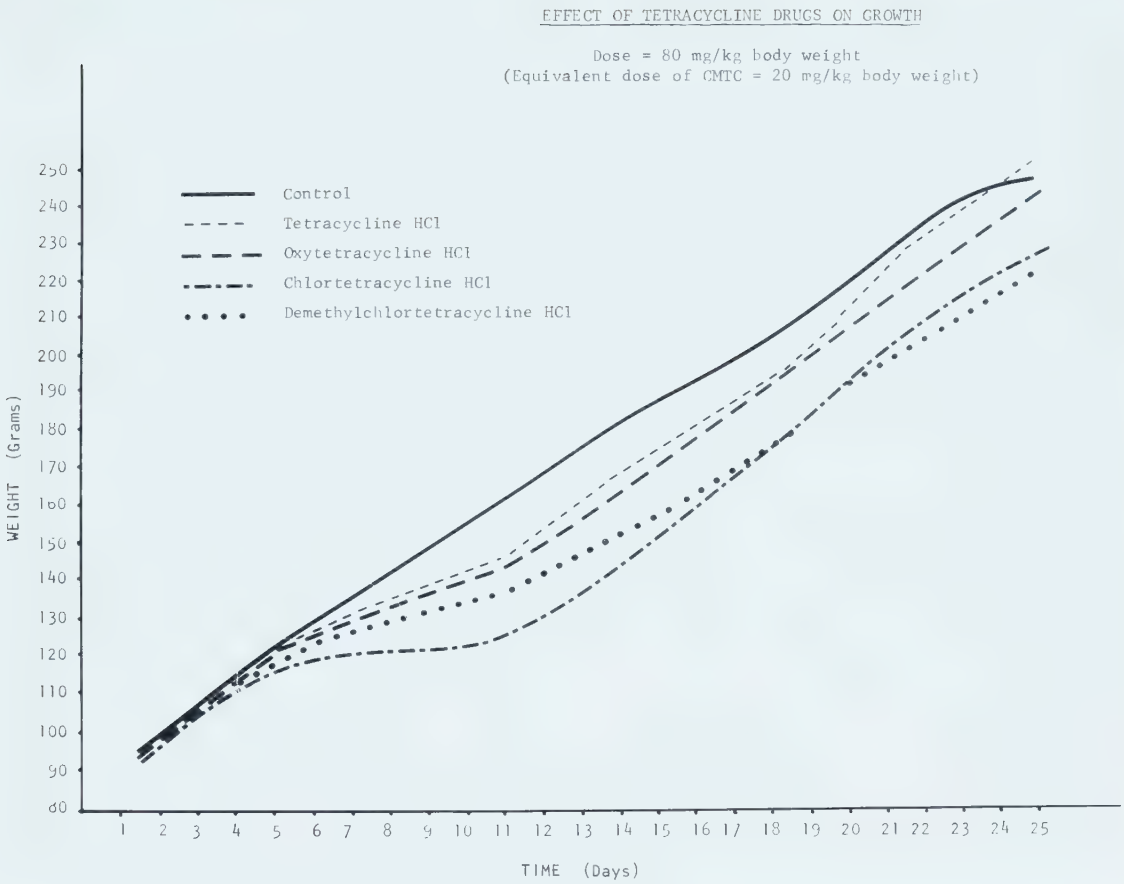

EFFECT OF TETRACYCLINE DRUGS ON GROWTH
Dose = 40 mg/kg body weight
(Equivalent dose of CMTC = 10 mg/kg body weight)
Control
Tetracycline HCl
Oxytetracycline HCl
Chlortetracycline HCl
Demethylchlortetracycline HCl
WEIGHT (Grams)
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
TIME (Days)

Figure 16

Figure 17:

Shows comparison of growth curves of rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in equivalent doses. (Dose given was 80 mg/kg body weight except for DCTC where the dose was 20 mg/kg body weight.) Total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period were given. Solid line indicates untreated controls.



EFFECT OF TETRACYCLINE DRUGS ON GROWTH

Dose = 80 mg/kg body weight
(Equivalent dose of CMTC = 20 mg/kg body weight)

Figure 17

Figure 18:

Shows comparison of growth curves of rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in equivalent doses. (Dose given was 200 mg/kg body weight except for DCTC where the dose was 50 mg/kg body weight.) Total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period were given. Solid line indicates the untreated controls.

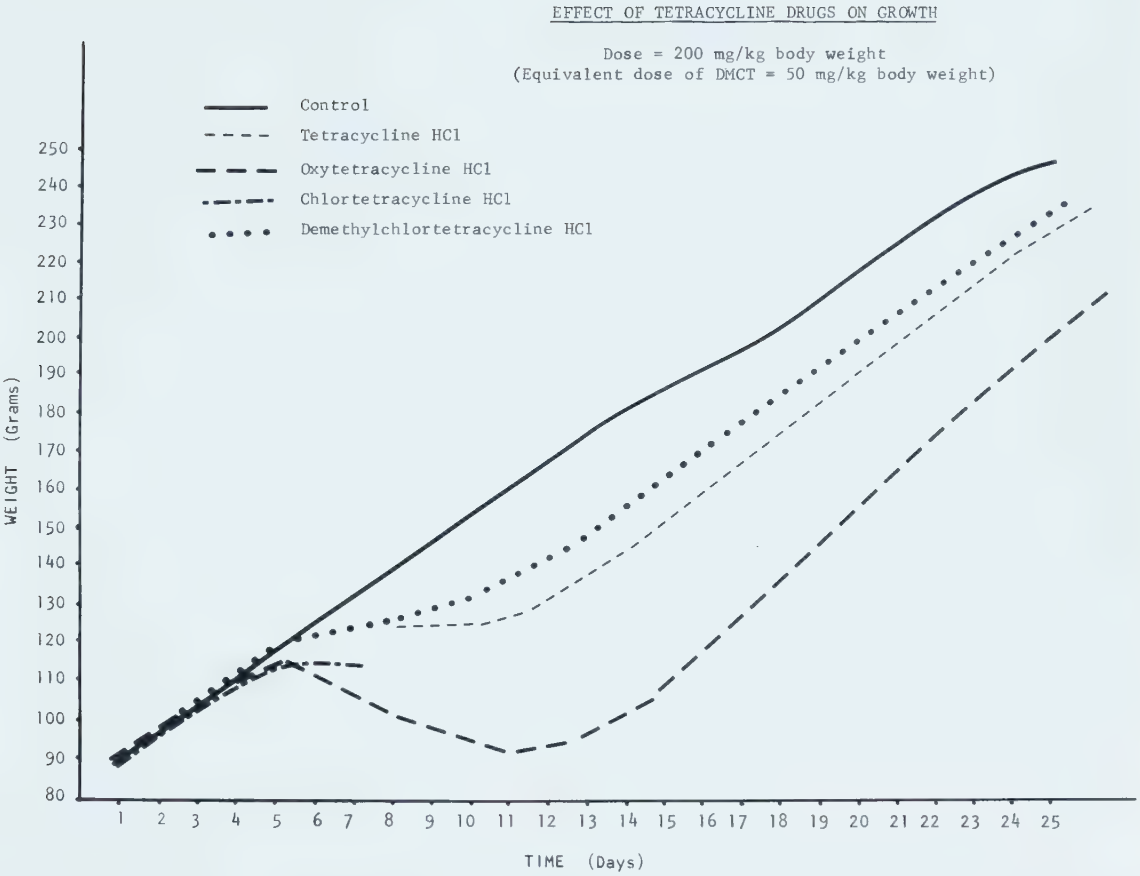

EFFECT OF TETRACYCLINE DRUGS ON GROWTH
Dose = 200 mg/kg body weight
(Equivalent dose of DMCT = 50 mg/kg body weight)
Control
Tetracycline HCl
Oxytetracycline HCl
Chlortetracycline HCl
Demethylchlortetracycline HCl
WEIGHT (Grams)
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
TIME (Days)

Figure 18

Figure 19:

A comparison of growth curves of untreated rats and rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in doses comparable on weight to weight basis (dose given is 10 mg/kg body weight). A total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period were given.

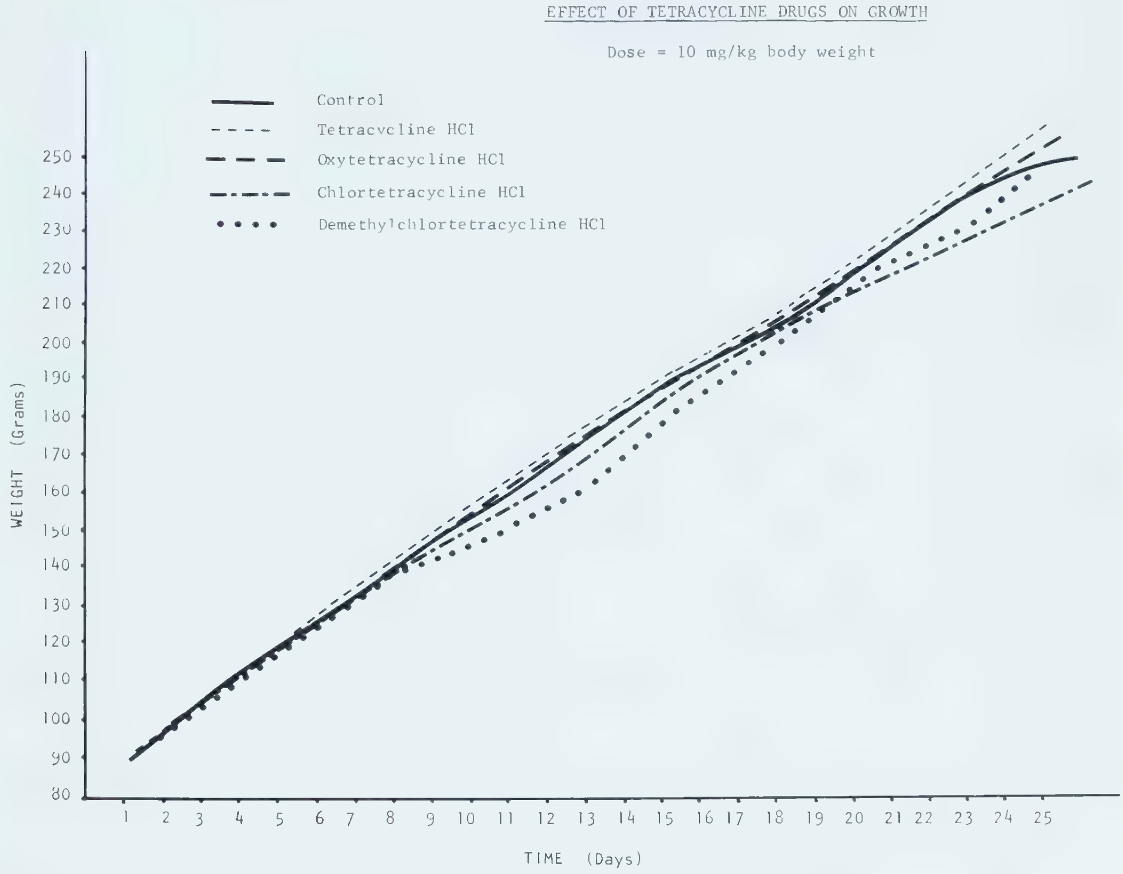

EFFECT OF TETRACYCLINE DRUGS ON GROWTH
Dose = 10 mg/kg body weight
Control
Tetracycline HCl
Oxytetracycline HCl
Chlortetracycline HCl
Demethylchlortetracycline HCl
WEIGHT (Grams)
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
TIME (Days)

Figure 19

Figure 20:

A comparison of growth curves of untreated rats and rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in doses comparable on weight to weight basis. (Dose given is 40 mg/kg body weight except for DCTC where the dose was 50 mg/kg body weight.) A total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period were given.

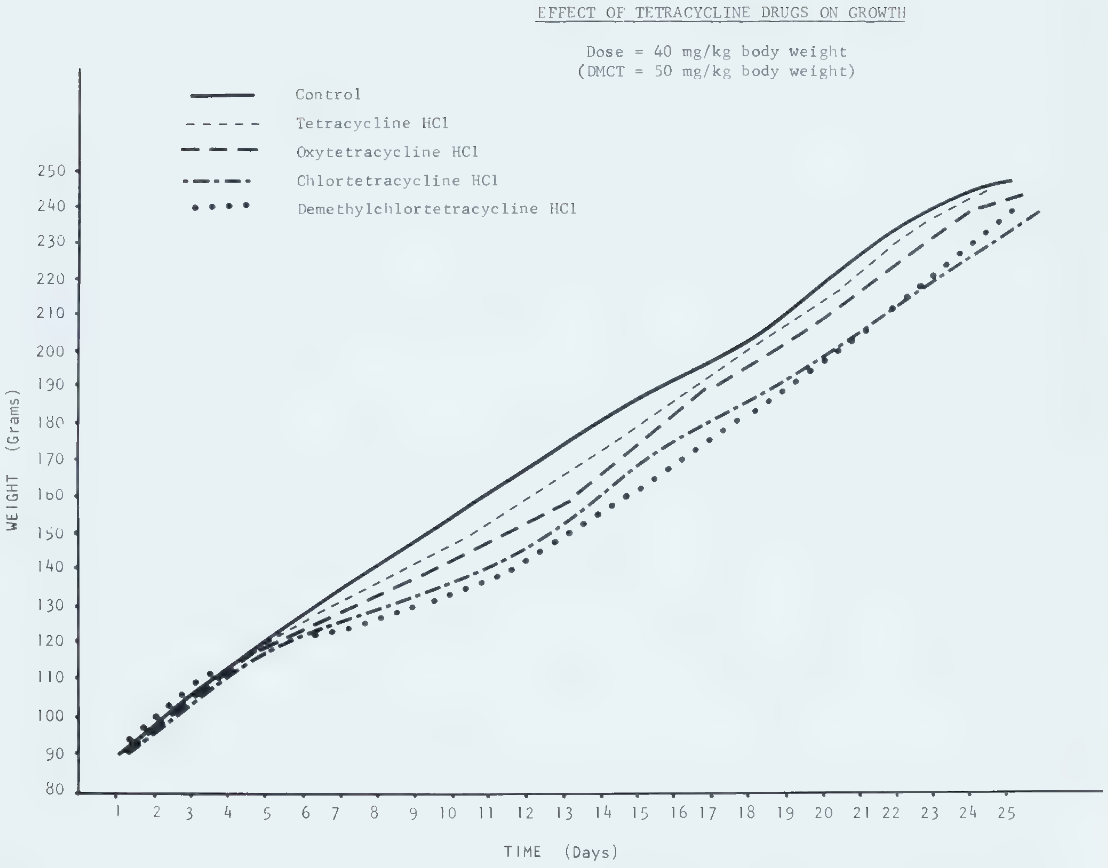

EFFECT OF TETRACYCLINE DRUGS ON GROWTH
Dose = 40 mg/kg body weight
(DMCT = 50 mg/kg body weight)
Control
Tetracycline HCl
Oxytetracycline HCl
Chlortetracycline HCl
Demethylchlortetracycline HCl
WEIGHT (Grams)
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
TIME (Days)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Figure 20

Figure 21:

A comparison of growth curves of untreated rats and rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in doses comparable on weight to weight basis. (Dose given is 80 mg/kg body weight.) A total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period, were given.

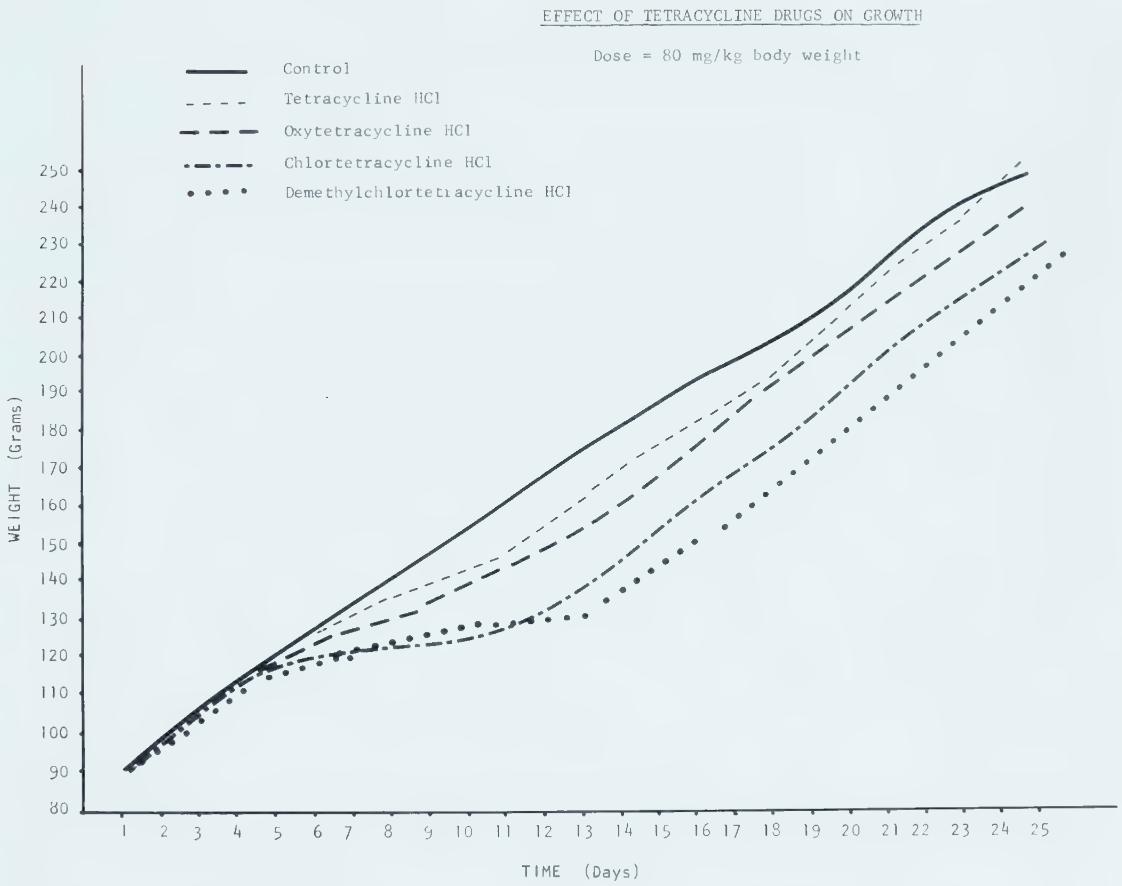

EFFECT OF TETRACYCLINE DRUGS ON GROWTH
Dose = 80 mg/kg body weight
Control
Tetracycline HCl
Oxytetracycline HCl
Chlortetracycline HCl
Demethylchlortetracycline HCl
WEIGHT (Grams)
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
TIME (Days)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Figure 21

Figure 22:

A comparison of growth curves of untreated rats and rats injected with four tetracycline drugs (TC, OTC, CTC and DCTC) in doses comparable on weight to weight basis. (Dose given is 200 mg/kg body weight except for DCTC where no such dose was employed.) Total of six injections, the first on the 5th and the sixth on the 10th day of the experimental period were given.

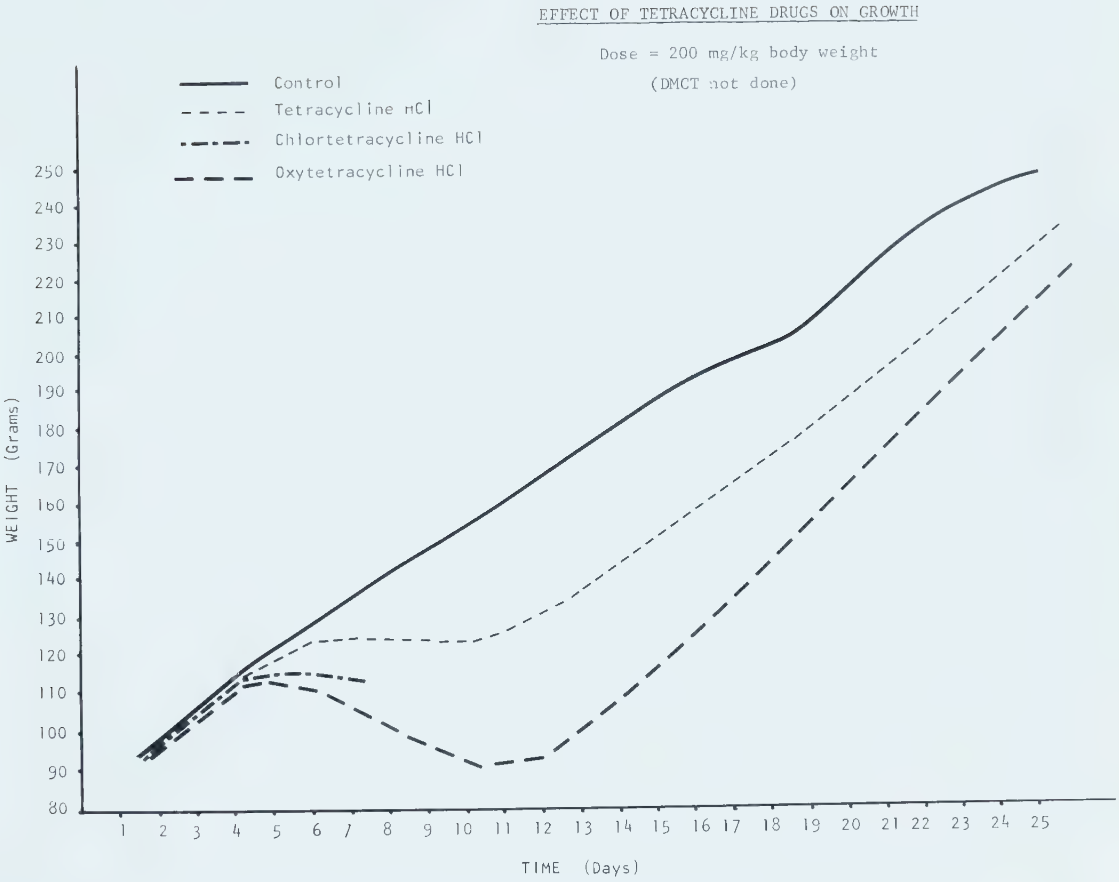

EFFECT OF TETRACYCLINE DRUGS ON GROWTH
Dose = 200 mg/kg body weight
(DMCT not done)
Control
Tetracycline HCl
Chlortetracycline HCl
Oxytetracycline HCl
WEIGHT (Grams)
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
TIME (Days)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Figure 22

TABLE X

LENGTHS OF FEMORA AND HUMERI OF RATS INJECTED WITH TETRACYCLINES AT DIFFERENT DOSE LEVELS

	FEMUR				HUMERUS			
	10[†]	40	80	200	10	40	80	200
Physiologic Saline	29.98[**]	29.98	29.98	29.98	24.46	24.46	24.46	24.46
Tetracycline	30.49	29.96	30.44	29.60	24.69	24.34	24.65	23.69
Oxytetracycline	30.50	30.16	30.47	28.35	24.53	24.15	24.43	22.97
Chlortetracycline	30.83	30.34	30.11	-	24.72	24.62	23.95	-
Demethylchlor-tetracycline[*]	30.44	30.69	30.16	30.28	24.77	24.73	24.62	24.49

[*] DCTC doses were 1/4 of the doses used for the other three drugs.

[**] Bone length (mm)

[†] Dose = mg/kg body weight

TABLE XI

WEIGHTS OF FEMORA AND HUMERI OF RATS INJECTED WITH TETRACYCLINES AT DIFFERENT DOSE LEVELS

	FEMUR				HUMERUS			
	10†	40	80	200	10	40	80	200
Physiologic Saline	0.5123**	0.5123	0.5123	0.5123	0.2520	0.2520	0.2520	0.2520
Tetracycline	0.5352	0.4833	0.4867	0.4488	0.2554	0.2453	0.2323	0.2233
Oxytetracycline	0.5314	0.5000	0.5517	0.4690	0.2587	0.2286	0.2604	0.2117
Chlortetracycline	0.5223	0.5094	0.5158	-	0.2346	0.2328	0.2153	-
Demethylchlortetracycline*	0.5454	0.5311	0.5406	0.5360	0.2444	0.2367	0.2562	0.2465

* DCTC doses were 1/4 of the doses used for the other three drugs.
** Bone Weight (gm)
† Dose = mg/kg body weight

TABLE XII

VOLUMES OF FEMORA AND HUMERI OF RATS INJECTED WITH TETRACYCLINES AT DIFFERENT DOSE LEVELS

	FEMUR				HUMERUS			
	10†	40	80	200	10	40	80	200
Physiologic Saline	0.3926**	0.3926	0.3926	0.3926	0.1855	0.1855	0.1855	0.1855
Tetracycline	0.4232	0.3829	0.4012	0.3752	0.1966	0.1815	0.1895	0.1736
Oxytetracycline	0.3848	0.3616	0.3982	0.3490	0.1833	0.1717	0.1853	0.1615
Chlortetracycline	0.3873	0.3733	0.3845	-	0.1847	0.1843	0.1783	-
Demethylchlortetracycline*	0.4029	0.3885	0.3919	0.3904	0.1891	0.1867	0.1857	0.1858

* DCTC doses were 1/4 of the doses used for the other three drugs.

** Volume (cm^3)

† Dose = mg/kg body weight

TABLE XIII

CALCULATED DENSITIES OF FEMORA AND HUMERI OF RATS INJECTED WITH TETRACYCLINES AT DIFFERENT DOSE LEVELS

	FEMUR				HUMERUS			
	10[†]	40	80	200	10	40	80	200
Physiologic Saline	1.303[**]	1.303	1.303	1.303	1.357	1.357	1.357	1.357
Tetracycline	1.264	1.262	1.214	1.196	1.300	1.348	1.225	1.285
Oxytetracycline	1.380	1.382	1.384	1.343	1.410	1.331	1.404	1.304
Chlortetracycline	1.348	1.365	1.341	-	1.269	1.263	1.207	-
Demethylchlor-tetracycline	1.348	1.368	1.379	1.373	1.292	1.267	1.379	1.327

[*] DCTC doses were 1/4 of the doses used for the other three drugs.
[**] Density
[†] Dose = mg/kg body weight

TABLE XIV

WEIGHTS OF ORGANIC AND INORGANIC CONSTITUENTS OF FEMORA AND HUMERI OF RATS INJECTED WITH TETRACYCLINE HCl AT DIFFERENT DOSE LEVELS

	FEMUR					HUMERUS				
	Control	10[†]	40	80	200	Control	10	40	80	200
A Weight before decalcification (organic + inorganic)	0.5027*	0.5437	0.4693	0.4935	0.4504	0.2395	0.2569	0.2443	0.2339	0.2297
B Weight after decalcification (organic)	0.3416	0.3815	0.3358	0.3847	0.3661	0.1654	0.1786	0.1614	0.1899	0.1757
Weight difference (A - B = inorganic)	0.1612	0.1622	0.1335	0.1088	0.0843	0.0741	0.0783	0.0829	0.0440	0.0540
Inorganic (%)	32.07	29.83	28.45	22.05	18.72	30.93	30.48	33.93	18.81	23.50

* Weight (gm)

[†] Dose = mg/kg body weight

TABLE XV

WEIGHTS OF ORGANIC AND INORGANIC CONSTITUENTS OF FEMORA AND HUMERI OF RATS INJECTED WITH OXYTETRACYCLINE HCl AT DIFFERENT DOSE LEVELS

	FEMUR					HUMERUS				
	Control	10†	40	80	200	Control	10	40	80	200
A Weight before decalcification (organic + inorganic)	0.5027*	0.5323	0.4978	0.5254	0.4690	0.2395	0.2611	0.2322	0.2551	0.2116
B Weight after decalcification (organic)	0.3416	0.3690	0.3453	0.3773	0.3582	0.1654	0.1824	0.1693	0.1809	0.1595
Weight difference (A - B = inorganic)	0.1612	0.1633	0.1525	0.1481	0.1108	0.0741	0.0788	0.0629	0.0742	0.0522
Inorganic (%)	32.07	30.68	30.63	28.19	23.62	30.93	30.18	27.09	29.09	24.70

* Weight (gm)

† Dose = mg/kg body weight

TABLE XVI

WEIGHTS OF ORGANIC AND INORGANIC CONSTITUENTS OF FEMORA AND HUMERI OF RATS INJECTED WITH CHLORTETRACYCLINE HCl AT DIFFERENT DOSE LEVELS

	FEMUR					HUMERUS				
	Control	10[†]	40	80	200	Control	10	40	80	200
A Weight before decalcification (organic + inorganic)	0.5027[*]	0.5235	0.5149	0.5072	-	0.2395	0.2219	0.2391	0.2093	-
B Weight after decalcification (organic)	0.3416	0.3847	0.3627	0.3697	-	0.1654	0.1857	0.1903	0.1770	-
Weight difference (A - B = inorganic)	0.1612	0.1388	0.1522	0.1375	-	0.0741	0.0372	0.0488	0.0323	-
Inorganic (%)	32.07	26.51	29.56	27.11	-	30.93	16.76	20.41	15.43	-

[*] Weight (gm)
[†] Dose = mg/kg body weight

TABLE XVII

WEIGHTS OF ORGANIC AND INORGANIC CONSTITUENTS OF FEMORA AND HUMERI OF RATS INJECTED WITH DEMETHYLCHLORTETRACYCLINE HCl AT DIFFERENT DOSE LEVELS

	FEMUR					HUMERUS				
	Control	2.5[†]	10	20	50	Control	2.5	10	20	50
A Weight before decalcification (organic + inorganic)	0.5027*	0.5542	0.5405	0.5340	0.5601	0.2395	0.2536	0.2446	0.2479	0.2536
B Weight after decalcification (organic)	0.3416	0.3929	0.3836	0.3766	0.4074	0.1654	0.1926	0.1928	0.1834	0.1916
Weight difference (A - B = inorganic)	0.1612	0.1613	0.1569	0.1574	0.1527	0.0741	0.0610	0.0518	0.0645	0.0620
Inorganic (%)	32.07	29.11	29.03	29.48	27.26	30.93	24.05	21.18	26.02	24.45

* Weight (gm)
† Dose = mg/kg body weight

TABLE XVIII

TYPES OF DISCOLORATION AND FLUORESCENT COLOR INTENSITY OF FEMORA AND HUMERI OF RATS INJECTED WITH TETRACYCLINE DRUGS AT DIFFERENT DOSE LEVELS

DRUGS		DOSES mg/kg BODY WEIGHT			
		10	40	80	200
Tetracycline	Visible Light	Light Grey	Light Brown Yellow	Dark Brown Yellow	Dark Brown Yellow
	Fluorescent Intensity	+	+++++	+++++ ++++	+++++ +++++
	U.V. Light	Whitish Blue	Medium Yellow	Yellow	Deep Orange Yellow
Oxytetracycline	Visible Light	Light Grey	Light Grey	Medium Grey	Grey
	Fluorescent Intensity	+	++	+++	++++
	U.V. Light	Whitish Blue	Whitish Blue	Whitish Yellow	Light Yellow
Chlortetracycline	Visible Light	Light Grey	Light Brown Yellow	Medium Yellow	Brown Yellow
	Fluorescent Intensity	++	++++	+++++ +	+++++ +++
	U.V. Light	Whitish Yellow	Light Yellow	Medium Yellow	Yellow
Demethylchlor-tetracycline*	Visible Light	Light Grey	Medium Brown Yellow	Brown Yellow	Dark Brown Yellow
	Fluorescent Intensity	++	+++++	+++++ ++	+++++ ++++
	U.V. Light	Whitish Yellow	Medium Yellow	Yellow	Deep Orange Yellow

* DCTC doses were 1/4 of the doses used for the other three drugs.

TABLE XIX

PHOTOELECTRIC FLUORESCENT LIGHT EMISSION MEASUREMENTS OF FEMORA AND HUMERI OF RATS INJECTED WITH TETRACYCLINE DRUGS AT DIFFERENT DOSE LEVELS

DOSES	DOSES mg/kg BODY WEIGHT			
	10	40	80	200
Physiologic Saline	1030	1030	1030	1030
Tetracycline HCl	810	704	594	560
Oxytetracycline HCl	899	806	770	695
Chlortetracycline HCl	808	769	700	653
Demethylchlor-tetracycline HCl*	821	794	743	670

Note: The above measurements are percent light emitted x 10 under U.V. irradiation.

* Doses used are 1/4 of other three drugs.

Figure 23:

Photoelectric fluorescent light emission measurements of Femora and Humeri of rats injected with tetracycline drugs at different dose levels.

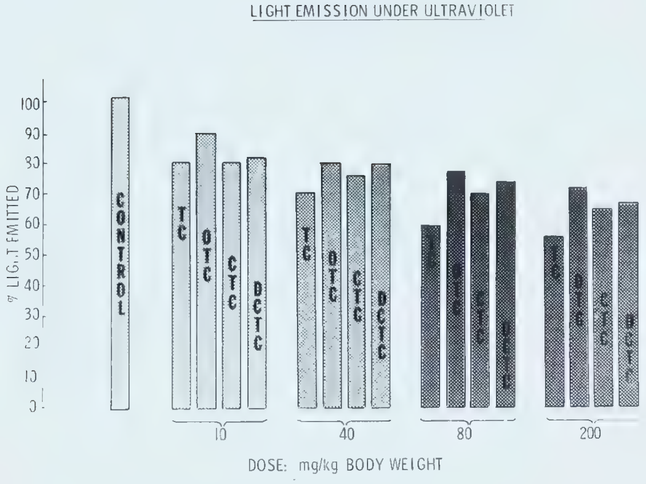

LIGHT EMISSION UNDER ULTRAVIOLET
% LIGHT EMITTED
100
90
80
70
60
50
40
30
20
10
0
CONTROL
TC
OTC
CTC
DCTC
10
40
80
200
DOSE: mg/kg BODY WEIGHT

Figure 23

TABLE XX

TYPE OF DISCOLORATION AND DEGREE OF FLUORESCENT COLOR INTENSITY OF MANDIBULAR AND MAXILLARY BONES OF RATS INJECTED WITH TETRACYCLINE DRUGS AT DIFFERENT DOSE LEVELS

DOSE: 10 mg/kg BODY WEIGHT

	Visible Light*		U.V. Light	
	Before Light Exposure	After Light Exposure	Before Light Exposure	After Light Exposure
TC	Light Brown +	Medium Brown ++	Whitish Yellow ++	Light Yellow ++
OTC	Light Gray -	Light Gray +	Whitish Blue +	Whitish Blue -
CTC	Light Gray -	Light Gray +	Whitish Blue +	Whitish Blue +
DCTC	Light Brown +	Medium Brown ++	Whitish Blue ++	Whitish Blue +

DOSE: 40 mg/kg BODY WEIGHT

TC	Brown Yellow +++	Bright Brown Yellow ++++	Medium Yellow +++++ +	Yellow +++++ ++
OTC	Light Brown +	Light Brown +	Whitish Blue ++	Whitish Blue +
CTC	Medium Brown Yellow ++	Brown Yellow +++	Whitish Yellow ++	Whitish Yellow +++
DCTC	Brown Yellow +++	Bright Brown Yellow ++++	Medium Yellow +++++ +	Bright Yellow +++++ +++

* Ordinary room lights

TABLE XX CONTINUED:

DOSE: 80 mg/kg BODY WEIGHT

	Visible Light*		U.V. Light	
	Before Light Exposure	After Light Exposure	Before Light Exposure	After Light Exposure
TC	Brown Yellow ++++	Bright Brown Yellow +++++	Yellow +++++ +++	Yellow +++++ ++++
OTC	Light Brown +	Light Brown +	Whitish Blue ++	Whitish Blue +
CTC	Light Brown +	Medium Brown ++	Medium Yellow ++++	Medium Yellow ++++
DCTC	Brown Yellow ++++	Brown Yellow +++++	Yellow +++++ +++	Bright Yellow +++++ ++++

DOSE: 200 mg/kg BODY WEIGHT

TC	Dark Brown Yellow +++++	Bright Orange Yellow +++++ +	Deep Orange Yellow +++++ ++++	Deep Yellow +++++ +++++
OTC	Medium Brown ++	Medium Brown Yellow ++	Medium Yellow ++++	Medium Yellow ++++
CTC	-	-	-	-
DCTC	Dark Brown Yellow +++++ +	Bright Orange Yellow +++++ ++	Deep Orange Yellow +++++ +++++	Deep Yellow +++++ +++++

* Ordinary room light

TABLE XXI

TYPE OF DISCOLORATION AND DEGREE OF FLUORESCENT COLOR INTENSITY OF MANDIBULAR AND MAXILLARY INCISOR TEETH OF RATS INJECTED WITH TETRACYCLINE DRUGS AT DIFFERENT DOSE LEVELS

DOSE: 10 mg/kg BODY WEIGHT

	Visible Light*		U.V. Light	
	Before Light Exposure	After Light Exposure	Before Light Exposure	After Light Exposure
TC	Light Brown +	Medium Yellow ++	Cream Yellow +	Cream Yellow ++
OCT	Light Gray -	Light Gray +	Whitish Blue -	Whitish Blue -
CTC	Light Gray +	Light Gray +	Cream Yellow +	Whitish Blue -
DCTC	Light Brown Yellow +	Medium Yellow ++	Whitish Blue -	Whitish Blue -

DOSE: 40 mg/kg BODY WEIGHT

TC	Brown Yellow +++	Bright Yellow ++++	Yellow ++++	Yellow +++++
OTC	Light Brown +	Light Brown +	Light Yellow ++	Light Yellow ++
CTC	Medium Brown Yellow ++	Orange Yellow ++++	Light Yellow ++	Light Yellow ++
DCTC	Brown Yellow ++	Bright Brown Yellow ++++	Yellow +++	Yellow ++++

* Ordinary room light

TABLE XXI CONTINUED:

DOSE: 80 mg/kg BODY WEIGHT

	Visible Light*		U.V. Light	
	Before Light Exposure	After Light Exposure	Before Light Exposure	After Light Exposure
TC	Brown Yellow ++++	Bright Brown Yellow +++++	Deep Orange Yellow +++++ +	Deep Orange Yellow +++++ ++
OTC	Light Brown +	Light Brown +	Yellow +++	Yellow +++
CTC	Light Brown +	Medium Yellow ++	Yellow +++	Yellow +++
DCTC	Brown Yellow +++	Brown Yellow +++++	Deep Orange Yellow +++++	Bright Orange Yellow +++++ +

DOSE: 200 mg/kg BODY WEIGHT

TC	Dark Brown Yellow +++++	Bright Orange Yellow +++++ +	Deep Orange Yellow +++++ +	Deep Orange Yellow +++++ ++
OTC	Medium Brown Yellow ++	Medium Brown Yellow ++	Yellow +++	Yellow +++
CTC	-	-	-	-
DCTD	Dark Brown Yellow +++++ +	Bright Orange Yellow +++++ ++	Deep Orange Yellow +++++ +	Deep Orange Yellow +++++ +

* Ordinary room light

Figure 24:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with tetracycline HCl at four different dose levels. From L to R: None, 10, 40, 80 and 200 mg/kg body weight.

Figure 25:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with oxytetracycline HCl at four different dose levels. From L to R: none, 10, 40, 80 and 200 mg/kg body weight.

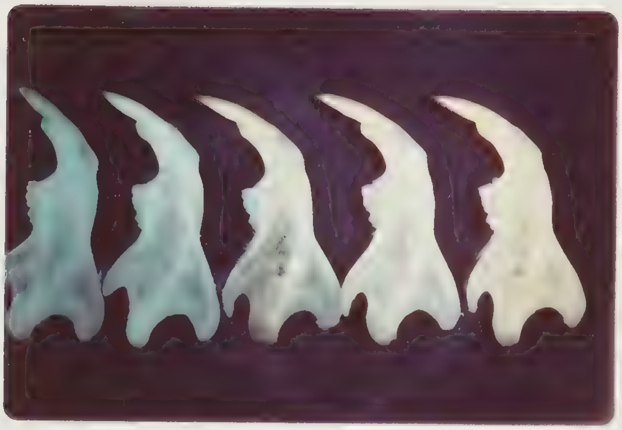


Figure 24

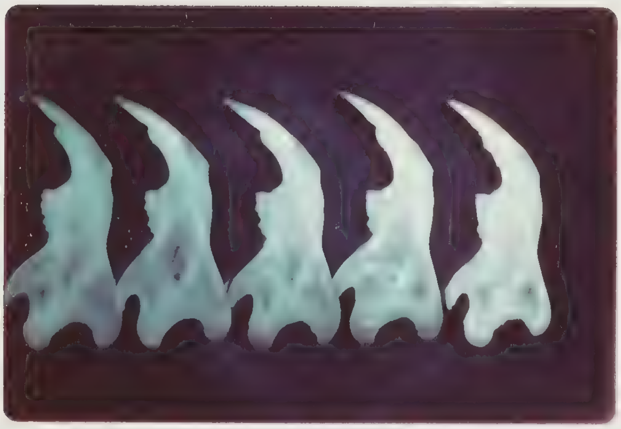


Figure 25

Figure 26:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with chlortetracycline HCl at four different dose levels. From L to R: none, 10, 40, 80 and 200 mg/kg body weight.

Figure 27:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with demethylchlortetracycline HCl at four different dose levels. From L to R: none, 2.5, 10, 20 and 50 mg/kg body weight.

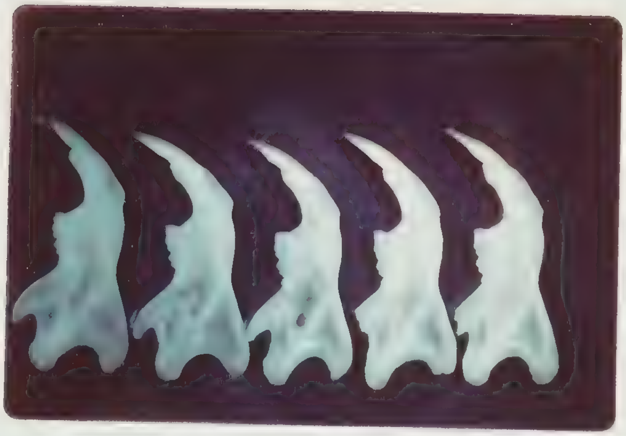


Figure 26

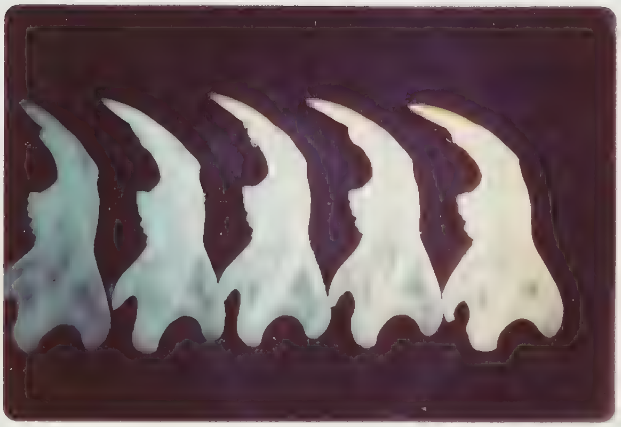


Figure 27

Figure 28:

Shows comparison of fluorescent color intensities of maxillary bones and teeth of rats injected with tetracycline HCl at four different dose levels. From L to R: none, 10, 40, 80 and 200 mg/kg body weight.

Figure 29:

Shows comparison of fluorescent color intensities of maxillary bones and teeth of rats injected with oxytetracycline HCl at four different dose levels. From L to R: none, 10, 40, 80 and 200 mg/kg body weight.

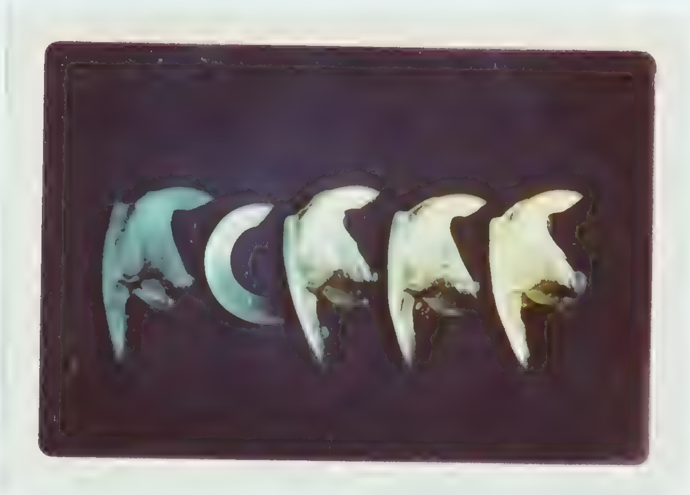


Figure 28

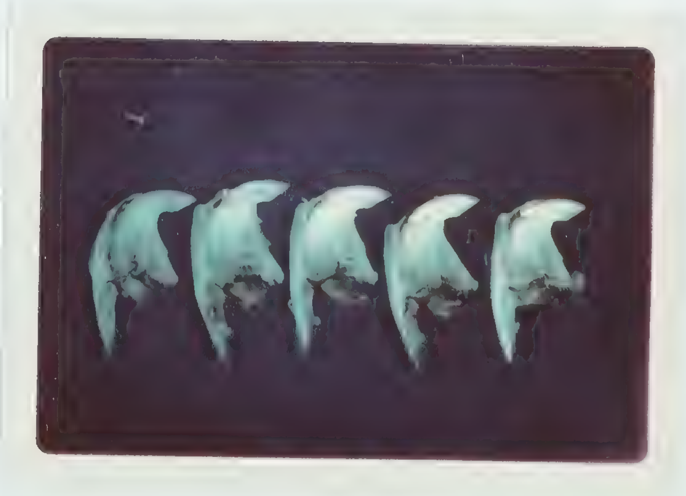


Figure 29

Figure 30:

Shows comparison of fluorescent color intensities of maxillary bones and teeth of rats injected with chlortetracycline HCl at four different dose levels. From L to R: none, 10, 40, 80 and 200 mg/kg body weight.

Figure 31:

Shows comparison of fluorescent color intensities of maxillary bones and teeth of rats injected with demethylchlortetracycline HCl at four different dose levels. From L to R: none, 2.5, 10, 20, 50 and 80 mg/kg body weight.

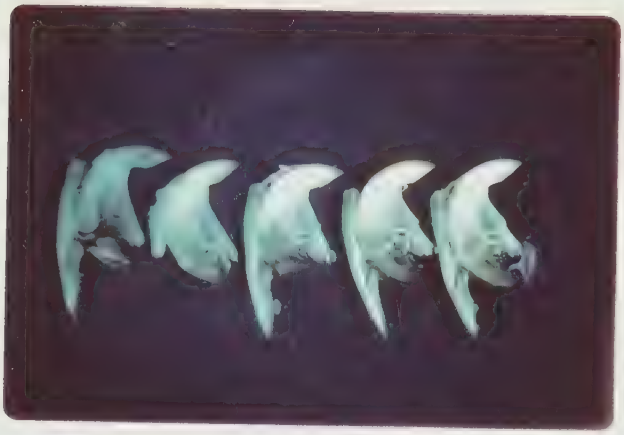


Figure 30

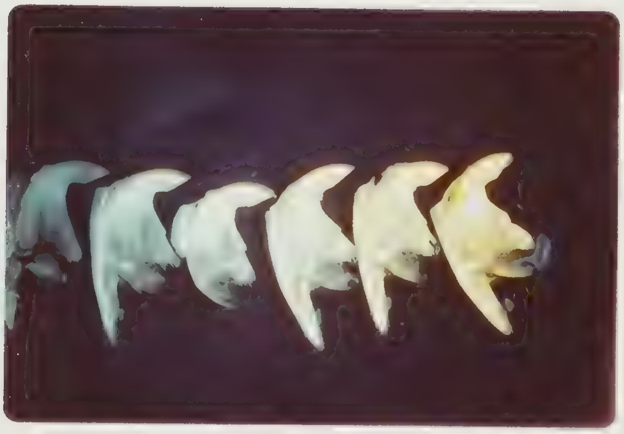


Figure 31

Figure 32:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with four different tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 10 mg/kg body weight dose level except for DCTC where the equivalent dose was 2.5 mg/kg body weight.

Figure 33:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with four different tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 40 mg/kg body weight dose level except for DCTC where the equivalent dose level was 10 mg/kg body weight.

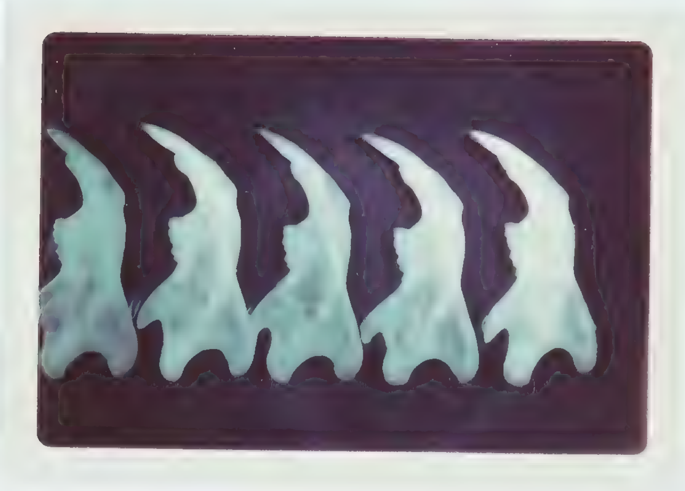


Figure 32

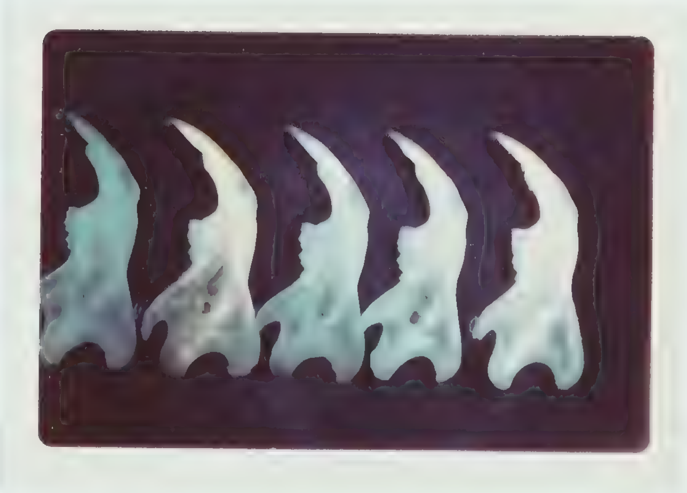


Figure 33

Figure 34:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with four different tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 80 mg/kg body weight dose level except for DCTC where the equivalent dose level was 20 mg/kg body weight.

Figure 35:

Shows comparison of fluorescent color intensities of mandibular bones and teeth of rats injected with four different tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 200 mg/kg body weight dose level except for DCTC where the equivalent dose level was 50 mg/kg body weight.

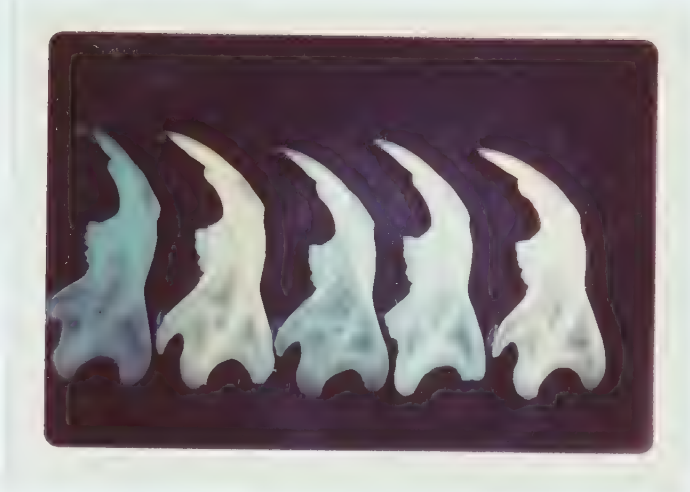


Figure 34

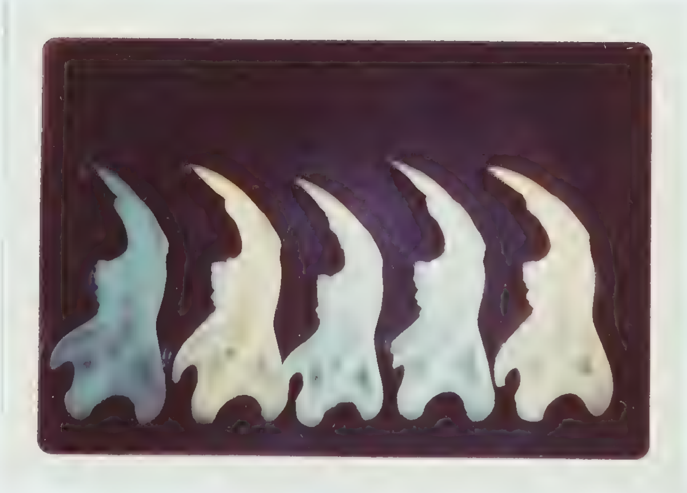


Figure 35

Figure 36:

Shows comparison of fluorescent color intensities of tibiae of rats injected with four tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 10 mg/kg body weight dose level except for DCTC where the equivalent dose level was 2.5 mg/kg body weight.

Figure 37:

Shows comparison of fluorescent color intensities of tibiae of rats injected with four tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 40 mg/kg body weight dose level except for DCTC where the equivalent dose level was 10 mg/kg body weight.

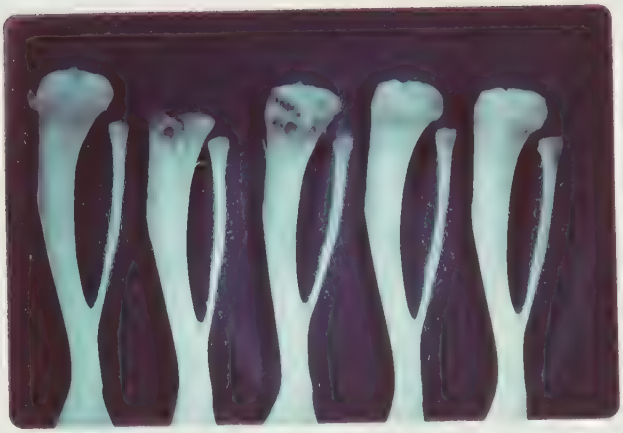


Figure 36

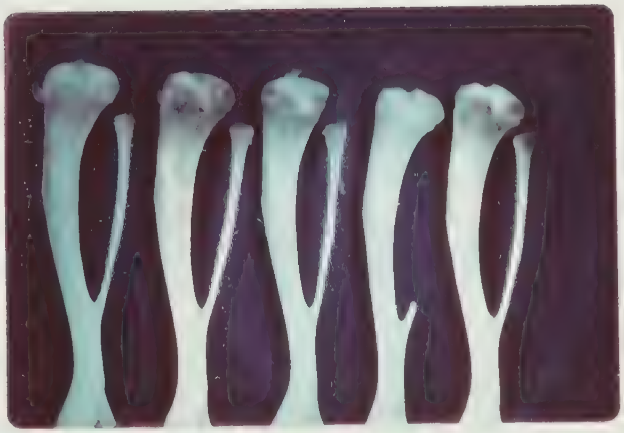


Figure 37

Figure 38:

Shows comparison of fluorescent color intensities of tibiae of rats injected with four tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 80 mg/kg body weight dose level except for DCTC where the equivalent dose level was 20 mg/kg body weight.

Figure 39:

Shows comparison of fluorescent color intensities of tibiae of rats injected with four tetracycline drugs. From L to R: control, TC, OTC, CTC and DCTC at 200 mg/kg body weight dose level except for DCTC where the equivalent dose level was 50 mg/kg body weight.

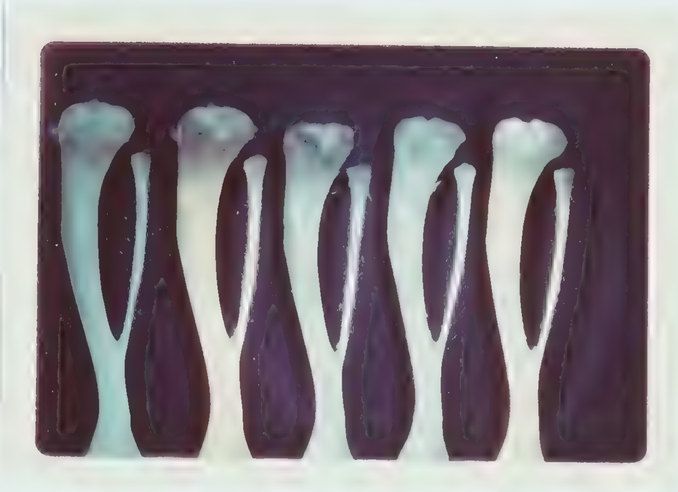


Figure 38

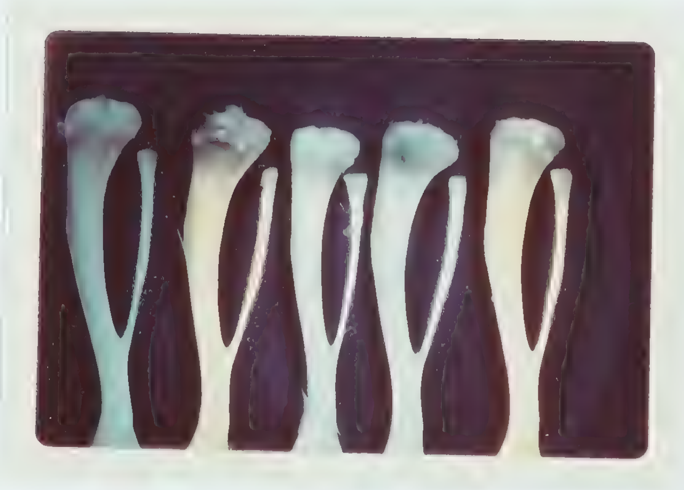


Figure 39

TABLE XXII

EVALUATION OF DISCOLORATION IN UNDECALCIFIED CROSS-SECTIONS OF RAT INCISOR TEETH GIVEN TETRACYCLINE DRUGS AT FOUR DIFFERENT DOSE LEVELS

Tetracycline HCl

Doses mg/kg	Type of Discoloration	Degree	Remarks
10	None	-	-Six discolored lines in dentine clear and distinct -No diffusion of drug in between the lines
40	Yellow	Mild	-Six discolored lines in dentine clear and distinct -No diffusion of drug between the lines
80	Yellow	Mild	-Discolored lines in dentine indistinct and unclear -Diffusion of drug between and pulpal to the lines present
200	Yellow	Moderate	-Discolored lines in dentine indistinct and unclear -Dentine formed during and after drug administration diffusely discolored -Discolored area of dentine appears to be wider

Oxytetracycline HCl

Doses mg/kg	Type of Discoloration	Degree	Remarks
10	None	-	-Six discolored lines in the dentine are clear -No diffusion of drug in between the lines
40	None	-	-Six discolored lines in the dentine are clear -No diffusion of drug in between the lines
80	None	-	-No discolored lines are seen
200	Yellow	Mild	-No discolored lines are seen -Area of dentine occupied by the lines appears to be wide

TABLE XXII CONTINUED:

Chlortetracycline HCl

Doses mg/kg	Type of Discoloration	Degree	Remarks
10	Yellow	Mild	-No definite discolored lines or area in dentine present
40	Yellow	Mild	-Six discolored lines in the dentine are clear -No diffusion of drug in between the lines
80	Yellow	Mild	-Faint indication that lines are present -Diffusion of drug in between the lines present
200	Yellow	Mild	-No definite discolored lines are seen -Area of dentine occupied by the lines appears to be wide -Dentine pulpal to the last dose appears dark

Demethylchlortetracycline HCl

Doses mg/kg	Discoloration	Degree	Remarks
2.5	None	-	-No discolored lines are seen in the dentine
10	Yellow	Mild	-No definite lines are present -Diffused discolored area in the dentine present
20	Yellow	Moderate	-Six discolored lines in the dentine are clear -Dentine pulpal to the last dosage is diffusely stained
50	Yellow	Severe	-Six lines are distinguishable -Dentine formed during and after drug administration periods diffusely stained

TABLE XXIII

EVALUATION OF U.V. FLUORESCENT COLOR INTENSITY IN UNDECALCIFIED CROSS SECTIONS OF RAT INCISOR TEETH GIVEN TETRACYCLINE DRUGS AT FOUR DIFFERENT DOSE LEVELS

Tetracycline HCl

Doses mg/kg	Type of Fluorescence	Degree	Remarks
10	Light Yellow	++	Enamel: -No fluorescence Dentine: -Fluorescent line clear and distinct. Alveolar Bone:
40	Yellow	++++	Enamel: -No fluorescence Dentine: -Fluorescent lines clear and very distinct. -No diffusion of the fluorescence in the adjoining area. Alveolar Bone: -Clearly marked cream yellow fluorescence in the region of Lamina dura.
80	Orange Yellow	+++++	Enamel: -No fluorescence Dentine: -Fluorescent lines clear -Diffusion of fluorescence in between the lines. -Dentine pulpal to the fluorescent lines also diffusely stained. Alveolar Bone:
200	Bright Orange Yellow	++++++	Enamel: -Slight yellow fluorescence Dentine: -Fluorescent lines not clearly marked. -The dentine formed during and after the drug administration periods is diffusely bright orange yellow -The area of dentine occupied by six fluorescent lines appears to be narrow.

TABLE XXIII CONTINUED:

Oxytetracycline HCl

Doses mg/kg	Type of Fluorescence	Degree	Remarks
10	Whitish Yellow	++	Enamel: -No fluorescence Dentine: -The fluorescent lines clear and very distinct -No diffusion of fluorescence in the adjoining areas Alveolar Bone: -Poorly marked cream white fluorescence in the region of lamina dura
40	Yellow	+++	Enamel: -No Fluorescence Dentine: -The fluorescent lines clear and very distinct -No diffusion of fluorescence in the adjoining areas Alveolar Bone: -Poorly marked cream white fluorescence in the region of lamina dura
80	Yellow	++++	Enamel: -No fluorescence Dentine: -The fluorescent lines clear and distinct -Diffusion of fluorescence between the lines -No staining of dentine pulpal to fluorescent lines Alveolar Bone: -Poorly marked cream white fluorescence in the region of lamina dura
200	Yellow	++++	Enamel: -Whitish yellow fluorescence Dentine: -No fluorescent lines can be distinguished -The area of dentine occupied by six fluorescent lines appears to be narrow -The dentine on both sides of the above area is stained slightly whitish yellow Alveolar Bone:

TABLE XXIII CONTINUED:

Chlortetracycline HCl

Doses mg/kg	Type of Fluorescence	Degree	Remarks
10	Whitish Gray	+	Enamel: -No fluorescence Dentine: -Fluorescent lines clear and distinct -No diffusion of fluorescence in the adjoining area Alveolar Bone:
40	Yellow	++++	Enamel: -No fluorescence Dentine: -Fluorescent lines clear and very distinct -No diffusion of fluorescence in the adjoining areas Alveolar Bone: -Poorly marked cream white fluorescence in the region of lamina dura
80	Yellow	+++++	Enamel: -Whitish yellow fluorescence Dentine: -Fluorescent lines clear and very distinct -No diffusion of fluorescence in the adjoining area Alveolar Bone: -Clearly marked cream yellow fluorescence in the region of lamina dura
200	Whitish Yellow	+++	Enamel: -Whitish yellow fluorescence Dentine: -First and sixth lines are only clearly fluorescent -Diffusion of fluorescence between the 1st and 6th line but no diffusion in the adjoining area Alveolar Bone:

TABLE XXIII CONTINUED:

Demethylchlortetracycline HCl

Doses mg/kg	Type of Fluorescence	Degree	Remarks
2.5	Whitish yellow	++	Enamel: -No fluorescence Dentine: -Fluorescent lines clear and very distinct -No diffusion of fluorescence between the lines Alveolar Bone: -Clearly marked cream white fluorescence in the region of lamina dura
10	Yellow	+++	Enamel: -No fluorescence Dentine: -Fluorescent lines are clear and very distinct -No diffusion of fluorescence in between the lines and the adjoining dentine
20	Yellow	++++	Enamel: -Whitish yellow fluorescence Dentine: -Fluorescent lines are clear and very distinct -No diffusion of fluorescence in between the lines and the adjoining dentine Alveolar Bone: -Very clearly marked yellowish fluorescence in the region of Lamina dura
50	Deep Orange Yellow	++++++	Enamel: -Dull whitish yellow fluorescence Dentine: -Fluorescent lines are clear and distinct -No diffusion of fluorescence in between the lines but some diffusion in dentine pulpal to 6th line Alveolar Bone: -Clearly marked orange yellow fluorescence in the bone
80	Deep Orange Yellow	+++++++	Enamel: -Light yellow fluorescence Dentine: -Fluorescent lines not clearly marked -The area of dentine occupied by six fluorescent lines appears to be narrow -Diffusion of fluorescence on both the alvealor and pulpal side of the line Alveolar Bone: -Clearly marked deep orange yellow fluorescence in the bone

Figure 40:

Shows whitish-blue autofluorescence in undecalcified cross sections of rat incisor in a control animal.

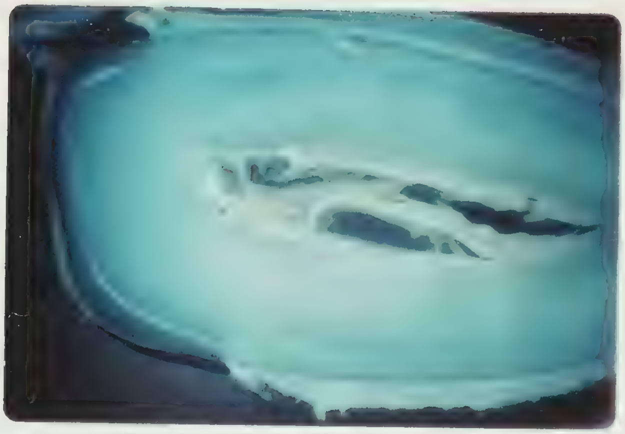


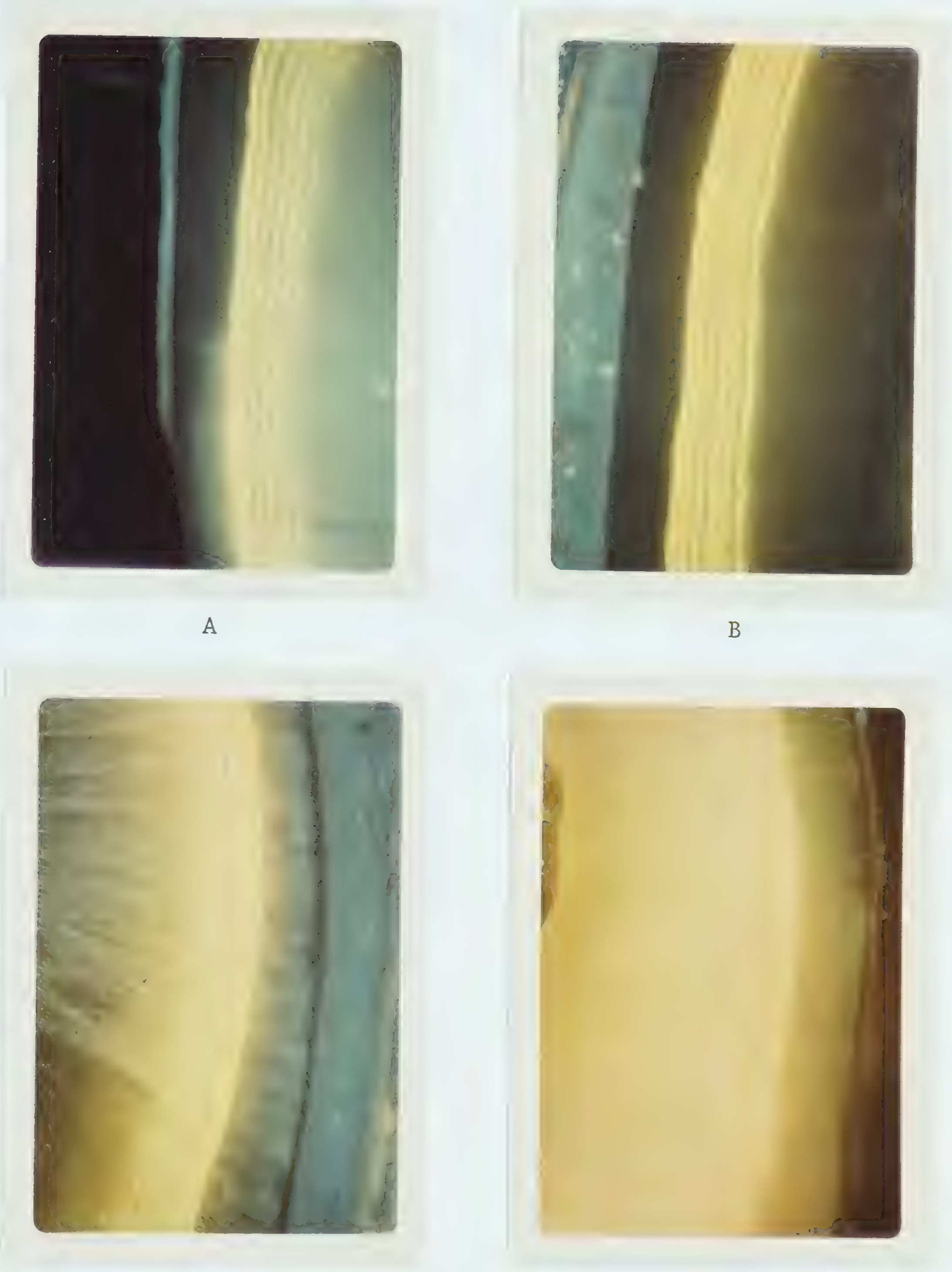
Figure 40

Figure 41:

Shows the extent and degree of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given tetracycline HCl at four different dose levels. Magnification x100. Exposure time 45 seconds.

A. Dose 10 mg/kg body weight
B. Dose 40 mg/kg body weight
C. Dose 80 mg/kg body weight
D. Dose 200 mg/kg body weight

NOTE: See Table XXIII for detailed descriptions of Figure 41.



A

B

C

D

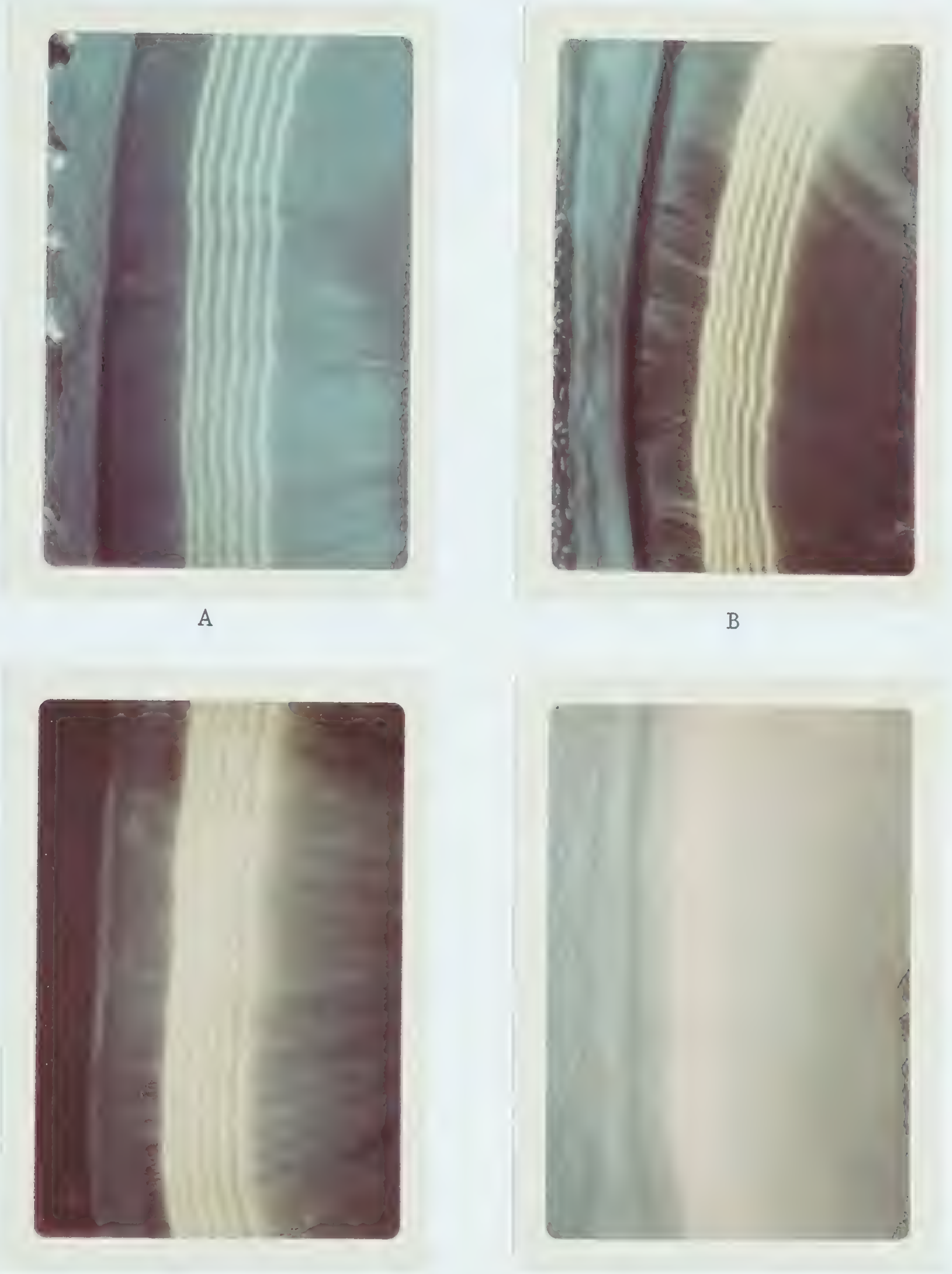
Figure 41

Figure 42:

Shows the extent and degree of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given oxytetracycline HCl at four different dose levels. Magnification x100. Exposure time 45 seconds.

A. Dose 10 mg/kg body weight
B. Dose 40 mg/kg body weight
C. Dose 80 mg/kg body weight
D. Dose 200 mg/kg body weight

NOTE: See Table XXIII for detailed description of Figure 42.



A

B

C

D

Figure 42

Figure 43:

Shows the extent and degree of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given chlortetracycline HCl at four different dose levels. Magnification x100. Exposure time 45 seconds.

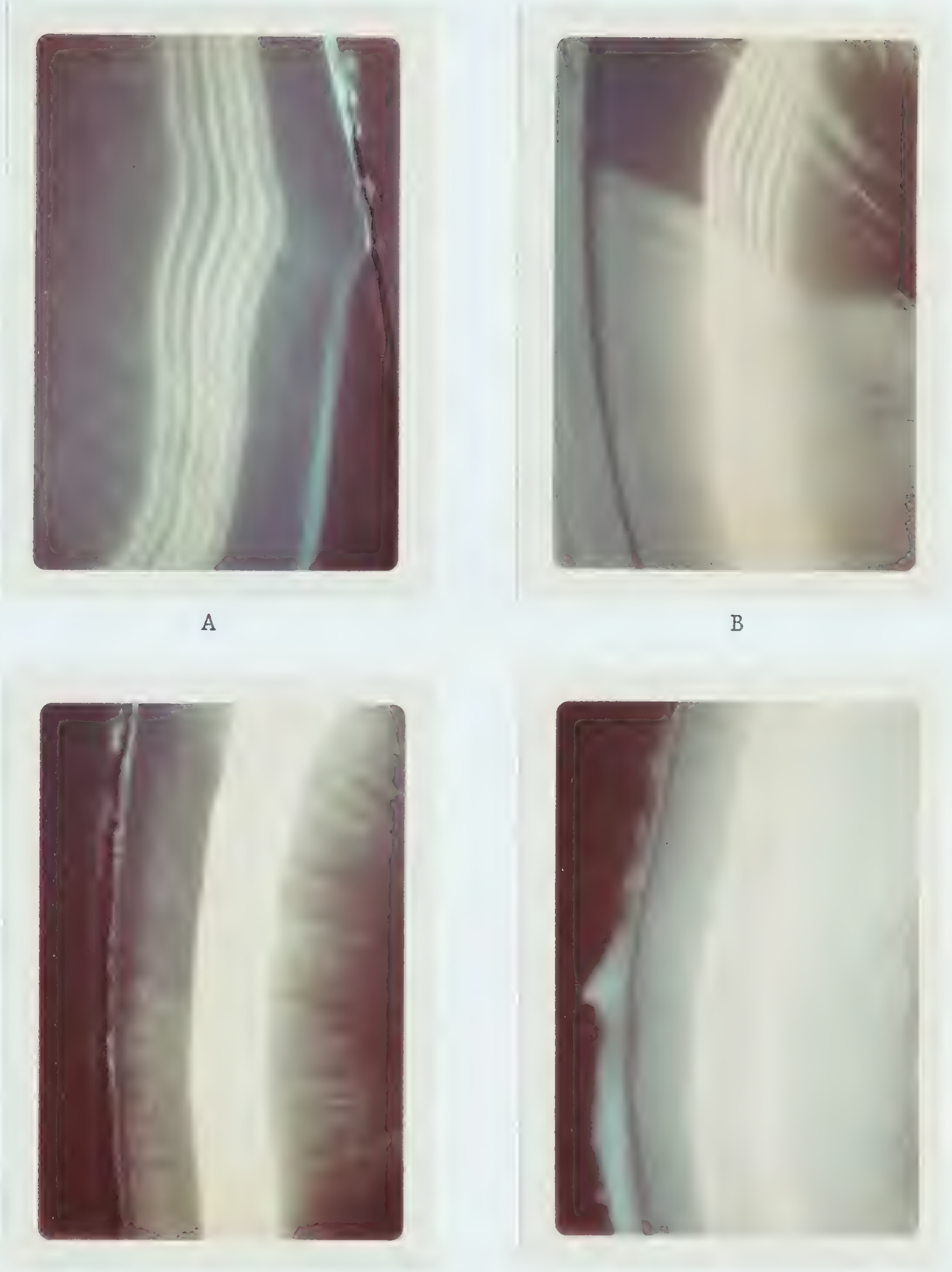
A. Dose 10 mg/kg body weight

B. Dose 40 mg/kg body weight

C. Dose 80 mg/kg body weight

D. Dose 200 mg/kg body weight

NOTE: See Table XXIII for detailed description of Figure 43.



A

B

C

D

Figure 43

Figure 44:

Shows the extent and degree of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given demethylchlortetracycline HCl at four different dose levels. Magnification x100. Exposure time 45 seconds.

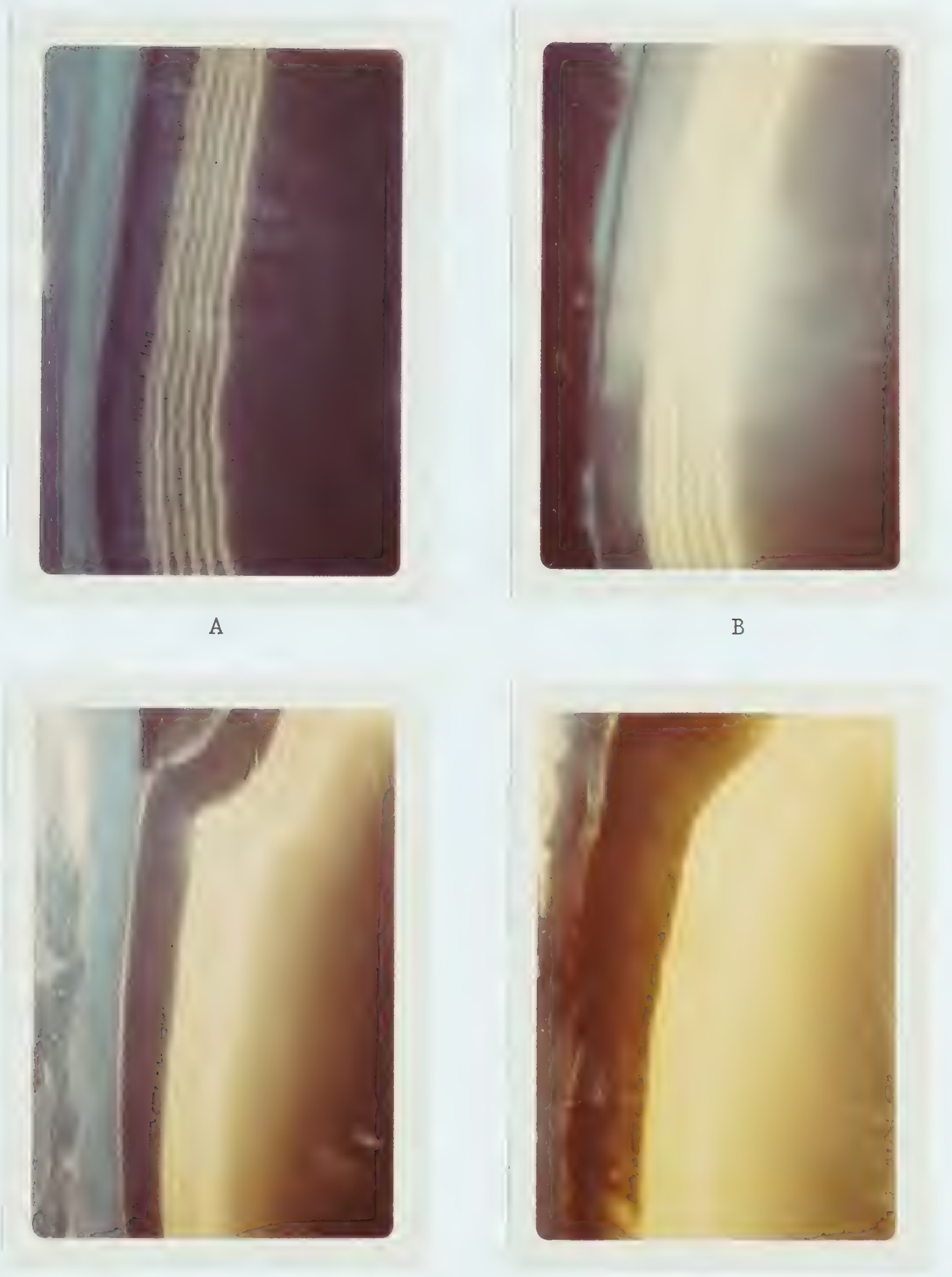
A. Dose 2.5 mg/kg body weight

B. Dose 10 mg/kg body weight

C. Dose 20 mg/kg body weight

D. Dose 50 mg/kg body weight

NOTE: See Table XXIII for detailed description of Figure 44.



A

B

C

D

Figure 44

Figure 45:

Shows the extent and degree of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given demethylchlortetracycline HCl at 80 mg/kg body weight dose level. Exposure time 45 seconds.

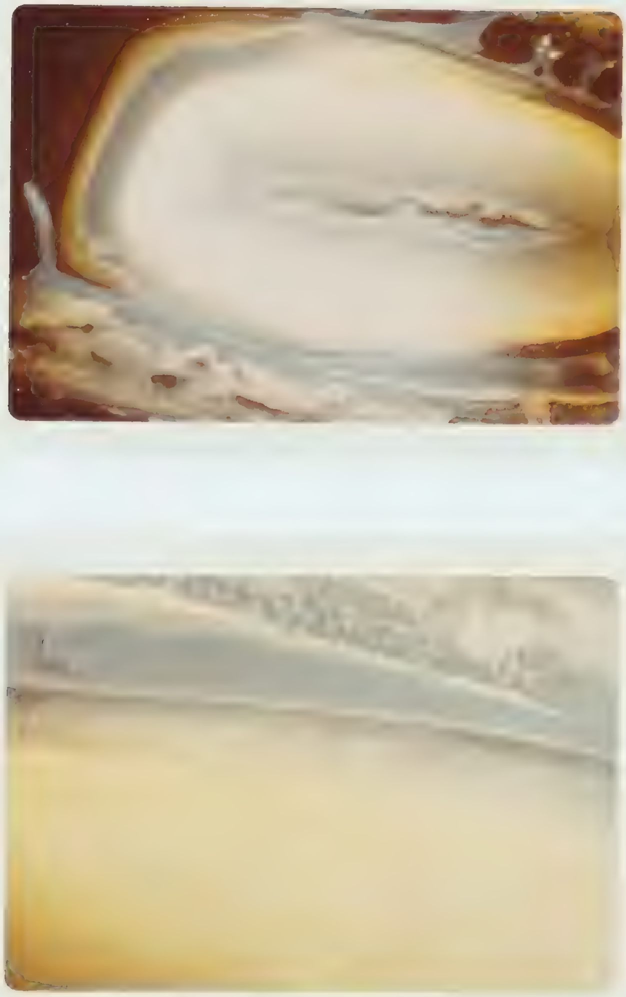


Figure 45

Figure 46:

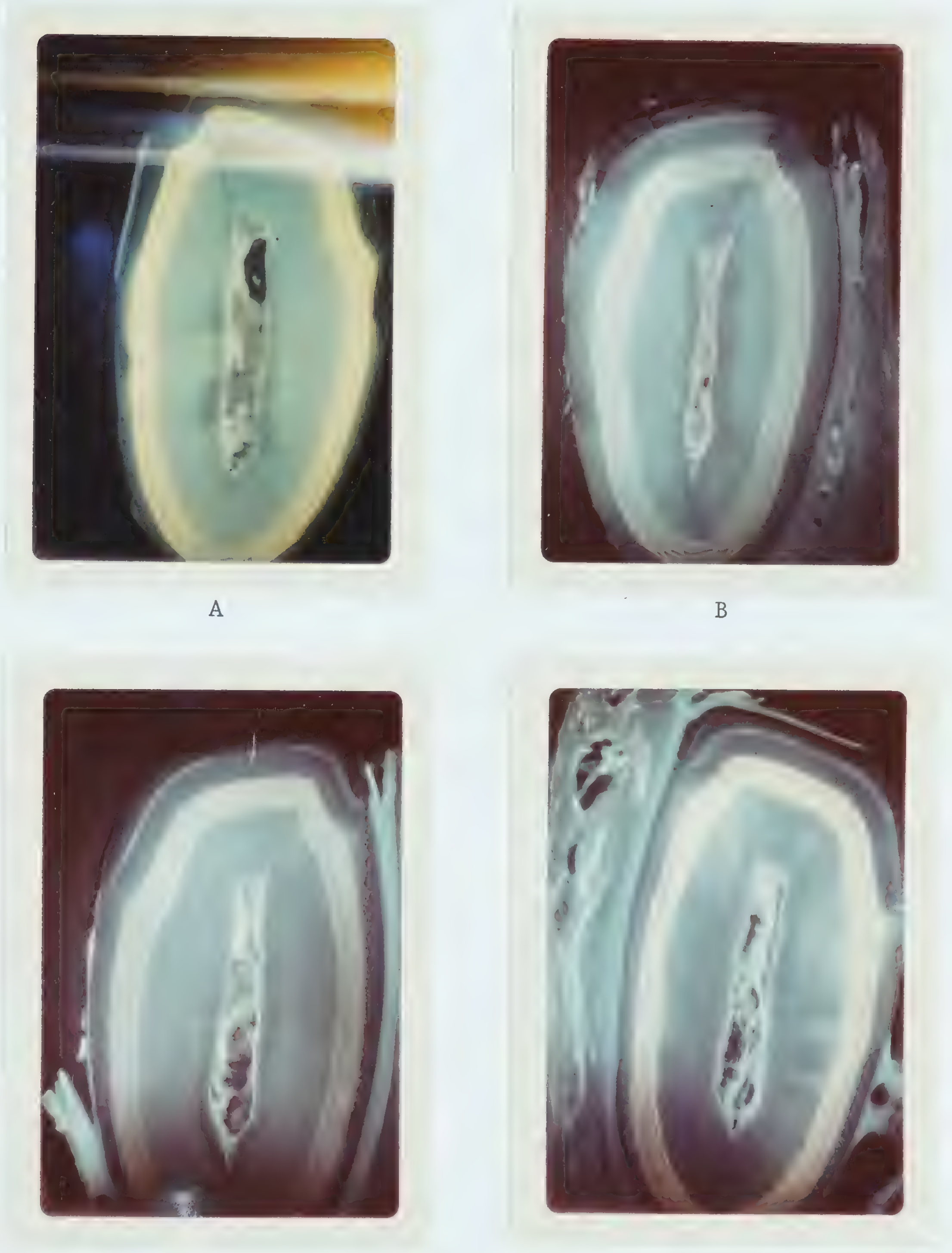
Shows comparison of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given four different tetracycline drugs at 10 mg/kg body weight dose level (DCTC equivalent dose was 2.5 mg/kg body weight). Magnification x35. Exposure time 60 seconds.

A. (1) Drug used - tetracycline HCl (TC)
   (2) Fluorescence - light yellow ++
   (3) Fluorescent lines in dentine clear and distinct.

B. (1) Drug used - oxytetracycline HCl (OTC)
   (2) Fluorescence - whitish yellow ++
   (3) Fluorescent lines in dentine clear and very distinct. No fluorescence in the enamel.

C. (1) Drug used - chlortetracycline HCl (CTC)
   (2) Fluorescence - whitish grey +
   (3) Fluorescent lines on dentine clear and distinct. No fluorescence in the enamel.

D. (1) Drug used - demethylchlortetracycline HCl (DCTC)
   (2) Fluorescence - whitish yellow ++
   (3) Fluorescent lines in dentine clear and very distinct. No fluorescence in the enamel.



A

B

C

D

Figure 46

Figure 47:

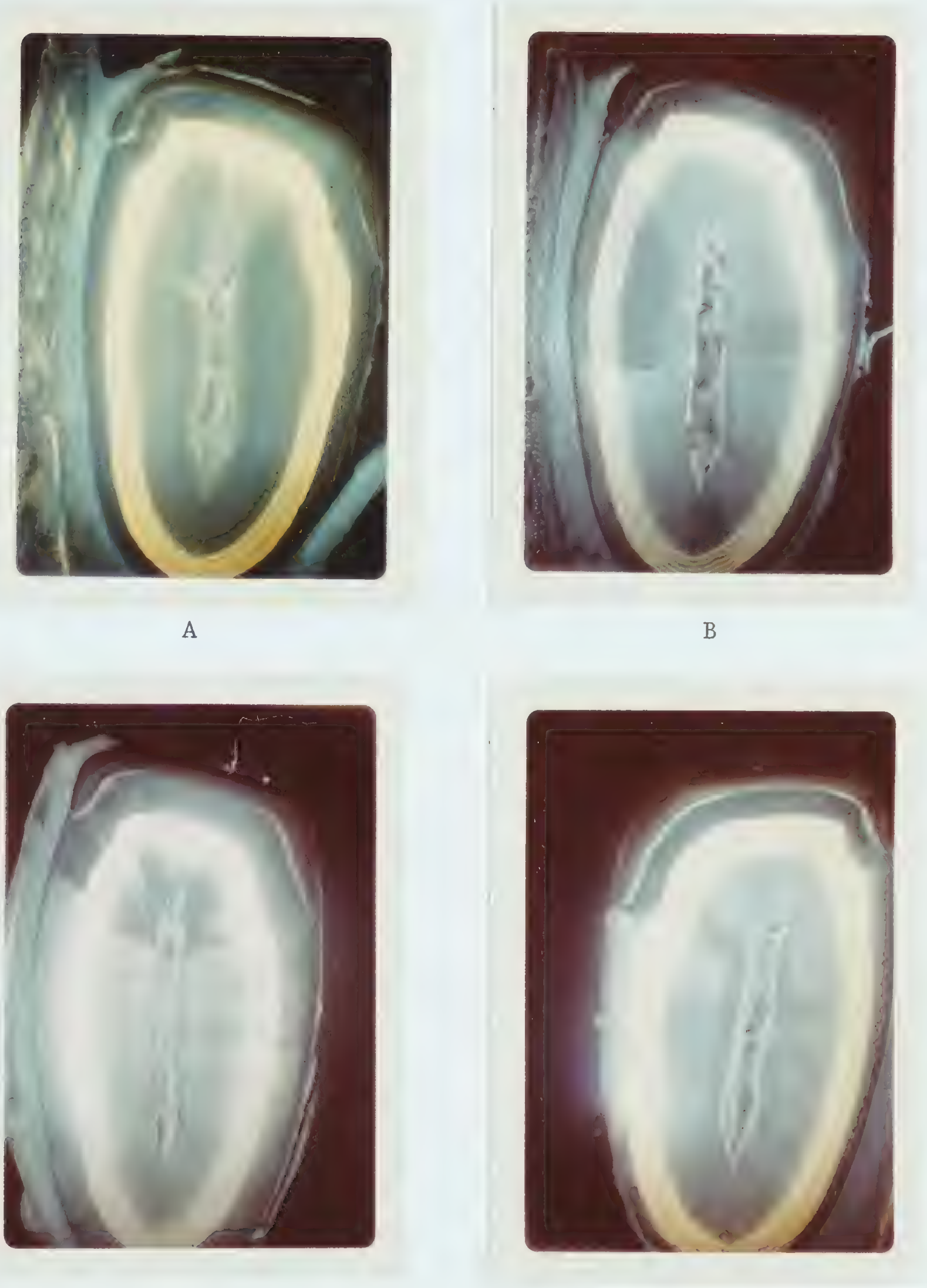
Shows comparison of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given four different tetracycline drugs at 40 mg/kg body weight dose level (DCTC equivalent dose was 10 mg/kg body weight). Magnification x35. Exposure time 60 seconds.

A. (1) Drug used - tetracycline HCl (TC)
   (2) Fluorescence - yellow ++++
   (3) Fluorescent lines in dentine clear and very distinct. No fluorescence in the enamel.

B. (1) Drug used - oxytetracycline HCl (OTC)
   (2) Fluorescence - yellow +++
   (3) Fluorescent lines in dentine clear and very distinct. No fluorescence in the enamel.

C. (1) Drug used - chlortetracycline HCl (CTC)
   (2) Fluorescence - yellow ++++
   (3) Fluorescent lines in dentine clear and very distinct. No fluorescence in the enamel.

D. (1) Drug used - demethylchlortetracycline HCl (DCTC)
   (2) Fluorescence - yellow +++
   (3) Fluorescent lines in dentine clear and very distinct. No fluorescence in the enamel.



A

B

C

D

Figure 47

Figure 48:

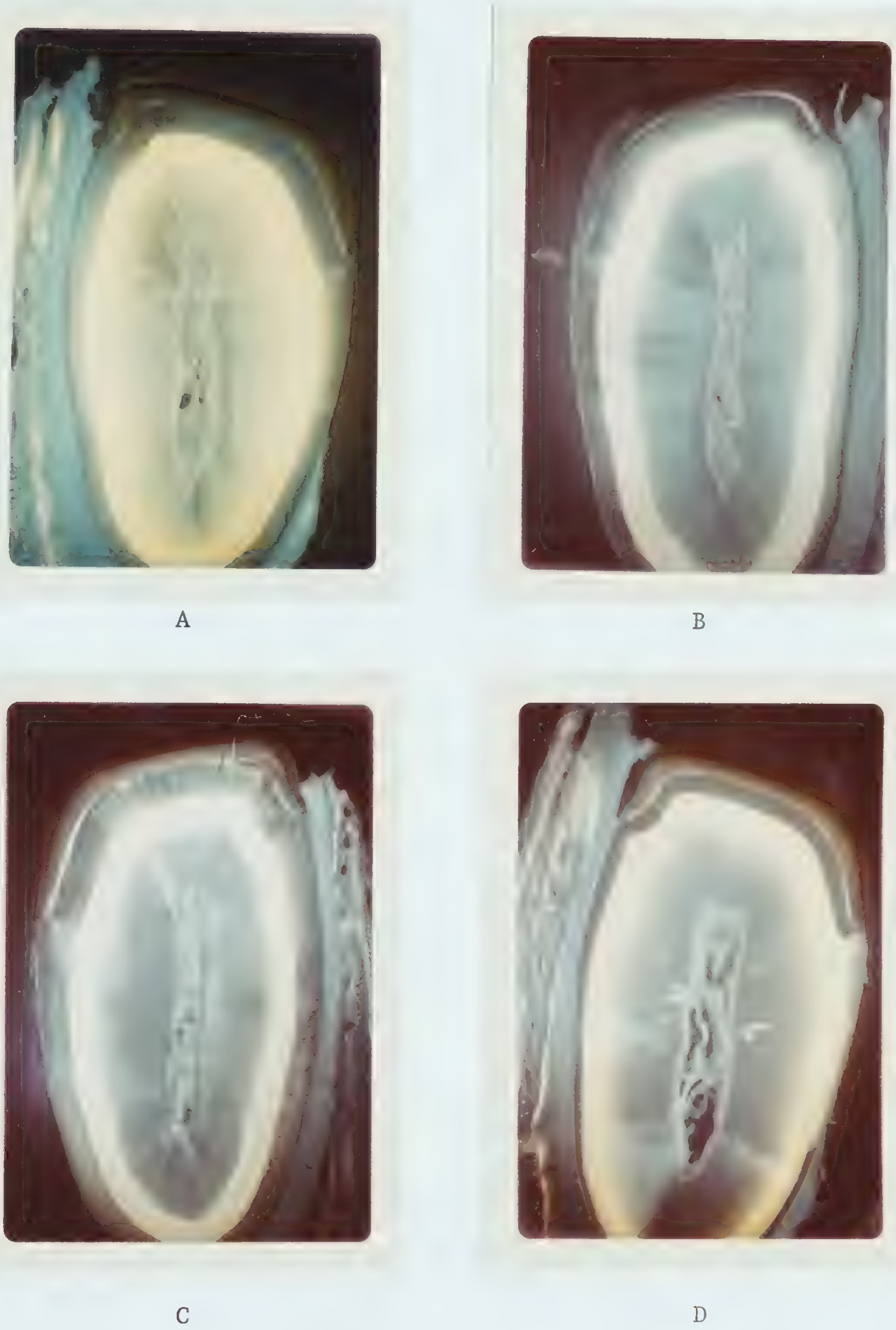
Shows comparison of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given four different tetracycline drugs at 80 mg/kg body weight dose level. (DCTC equivalent dose was 20 mg/kg body weight.) Magnification x35. Exposure time 60 seconds.

A. (1) Drug used - tetracycline HCl (TC)
   (2) Fluorescence - orange yellow ++++
   (3) Fluorescent lines in dentine clear. There is diffusion of fluorescence in between the lines and also in the area adjoining to the last fluorescent line. No fluorescence in the enamel.

B. (1) Drug used - oxytetracycline HCl (OTC)
   (2) Fluorescence - yellow ++++
   (3) Fluorescent lines clear and distinct. There is diffusion of fluorescence in between the lines but none in the area adjoining to the last fluorescent line. No fluorescence in the enamel.

C. (1) Drug used - chlortetracycline HCl (CTC)
   (2) Fluorescence - yellow ++++
   (3) Fluorescent lines clear and very distinct. There is no diffusion of fluorescence in between the lines and the adjoining areas of the dentine. Slight whitish yellow fluorescence is seen in the enamel.

D. (1) Drug used - demethylchlortetracycline HCl (DCTC)
   (2) Fluorescence - yellow ++++
   (3) Fluorescent lines clear and very distinct. There is no diffusion in between the lines and the adjoining areas of the dentine. Whitish yellow fluorescence is seen in the enamel.



A

B

C

D

Figure 48

Figure 49:

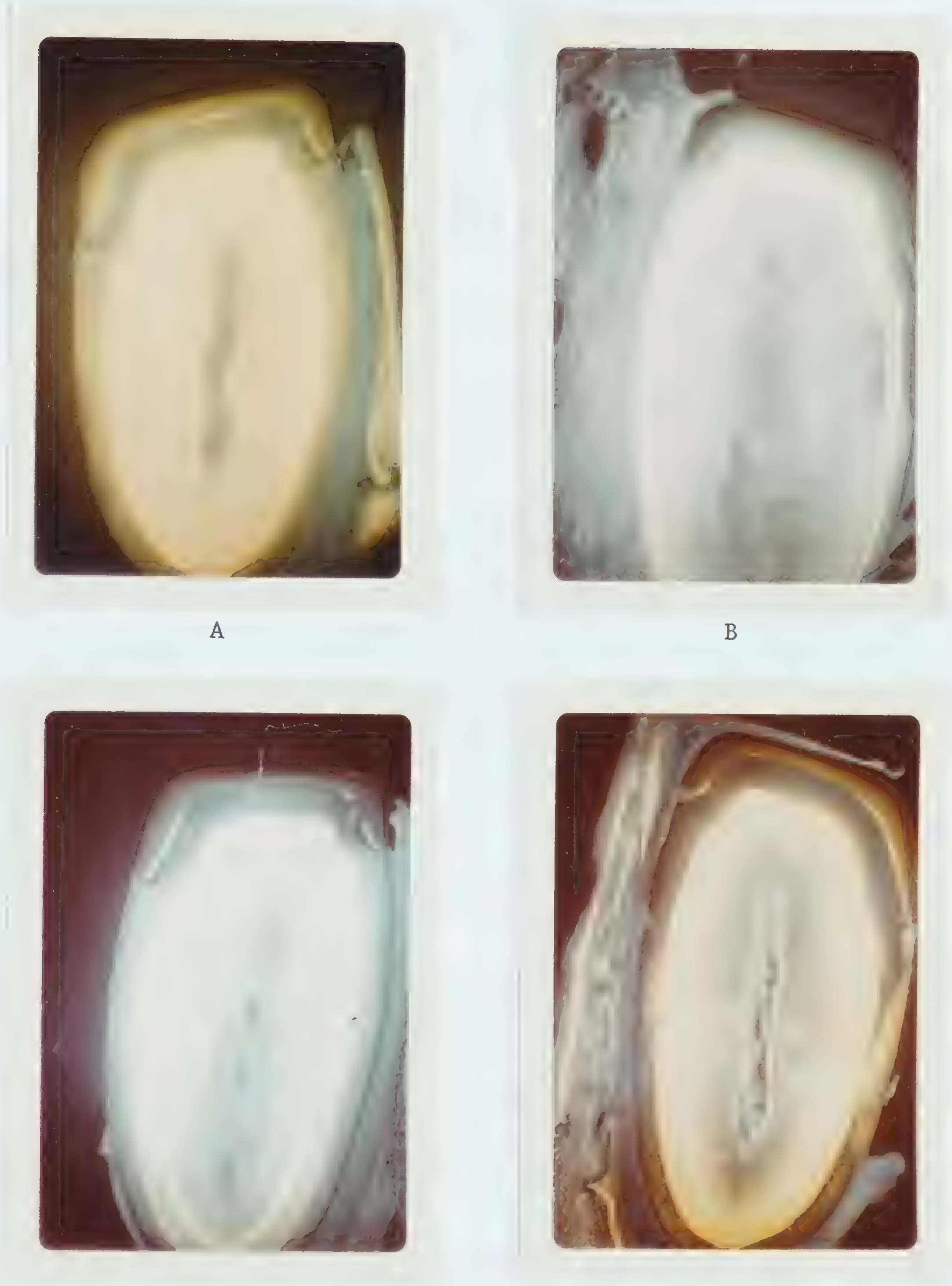
Shows comparison of U.V. fluorescent color intensity in undecalcified cross sections of rat incisor teeth given four different tetracycline drugs at 200 mg/kg body weight dose levels. (DCTC equivalent dose was 50 mg/kg body weight.) Magnification x35. Exposure time 60 seconds.

A. (1) Drug used - tetracycline HCl (TC)
  (2) Fluorescence - bright orange yellow $\underset{++}{++++}$
  (3) Fluorescent lines in dentine not clearly marked. The dentine formed during and after the drug administration periods shows diffuse fluorescence of bright orange yellow color. Slight yellow fluorescence present in the enamel.

B. (1) Drug used - oxytetracycline HCl (OTC)
  (2) Fluorescence - yellow ++++
  (3) Fluorescent lines in dentine not clearly marked. The dentine formed immediately before and after the drug administration periods show diffuse fluorescence of slight whitish yellow color. Whitish yellow fluorescence present in the enamel.

C. (1) Drug used - chlortetracycline HCl (CTC)
  (2) Fluorescence - whitish yellow +++
  (3) Fluorescent lines in dentine not clearly marked except the 1st and 6th line. There is diffusion of fluorescence between the lines but none in the area adjoining the lines. Whitish yellow fluorescence present in the enamel.

D. (1) Drug used - demethylchlortetracycline HCl (DCTC)
  (2) Fluorescence - deep orange yellow $\underset{++}{++++}$
  (3) Fluorescent lines in dentine clear and distinct. There is no diffusion of fluorescence in between the lines but some present in the area pulpal to the last line. Dull whitish yellow fluorescence present in the enamel.



A

B

C

D

Figure 49

TABLE XXIV

SUBJECTIVE EVALUATION OF THE MICRORADIOGRAPHS OF UNDECALCIFIED CROSS SECTIONS OF RAT INCISOR TEETH GIVEN TETRACYCLINE DRUGS AT FOUR DIFFERENT DOSE LEVELS

Tetracycline HCl

Doses mg/kg	Radiolucent Lines	Remarks
10	Present	-The radiolucent lines are faint. All the six lines are perceptible.
40	Present	-Very faint suggestion of radiolucent lines. Lines cannot be counted.
80	Present	-Very faint suggestion of radiolucent lines. Lines cannot be counted.
200	Present	-Very distinct wide radiolucent area in the dentine which appears to be pulpal to the faintly marked radiolucent lines.

Oxytetracycline HCl

10	Present	-Barely perceptible radiolucent lines present
40	Present	-Faintly marked radiolucent lines present.
80	Present	-Barely perceptible radiolucent lines present.
200	Present	-Very distinct wide radiolucent area in the dentine, more towards the pulp.

TABLE XXIV CONTINUED:

Chlortetracycline HCl

Doses mg/kg	Radiolucent Lines	Remarks
10	Present	-Clearly marked radiolucent lines present giving distinct banding effect.
40	Present	-Clearly marked radiolucent lines present.
80	Present	-Distinctly marked dark radiolucent lines present.
200	Present	-Very distinct wide radiolucent area in the dentine, more towards the pulp.

Demethylchlortetracycline HCl

2.5	Absent	-No radiolucent lines present
10	Present	-Faintly marked radiolucent lines present.
20	Present	-Faintly marked radiolucent lines present.
50	Present	-Very distinctly marked radiolucent lines present.

Figure 50:

Shows photographs and microradiographs of the ground section of rat incisor teeth given tetracycline drugs at 200 mg/kg body weight dose level. Magnification x35.

A. Photomicrograph of coronal part of the incisor.

  (1) Drug used - tetracycline HCl (TC)

  (2) Dentine shows discolored brown lines

  (3) Discolored lines correspond to the fluorescent lines (Fig. 49A)

B. Microradiograph of the same section and area shown in A.

  (1) Very distinct wide radiolucent area present in the dentine which appears more prominent towards the pulp in respect to other radiolucent lines.

C. Microphotograph of coronal part of the incisor.

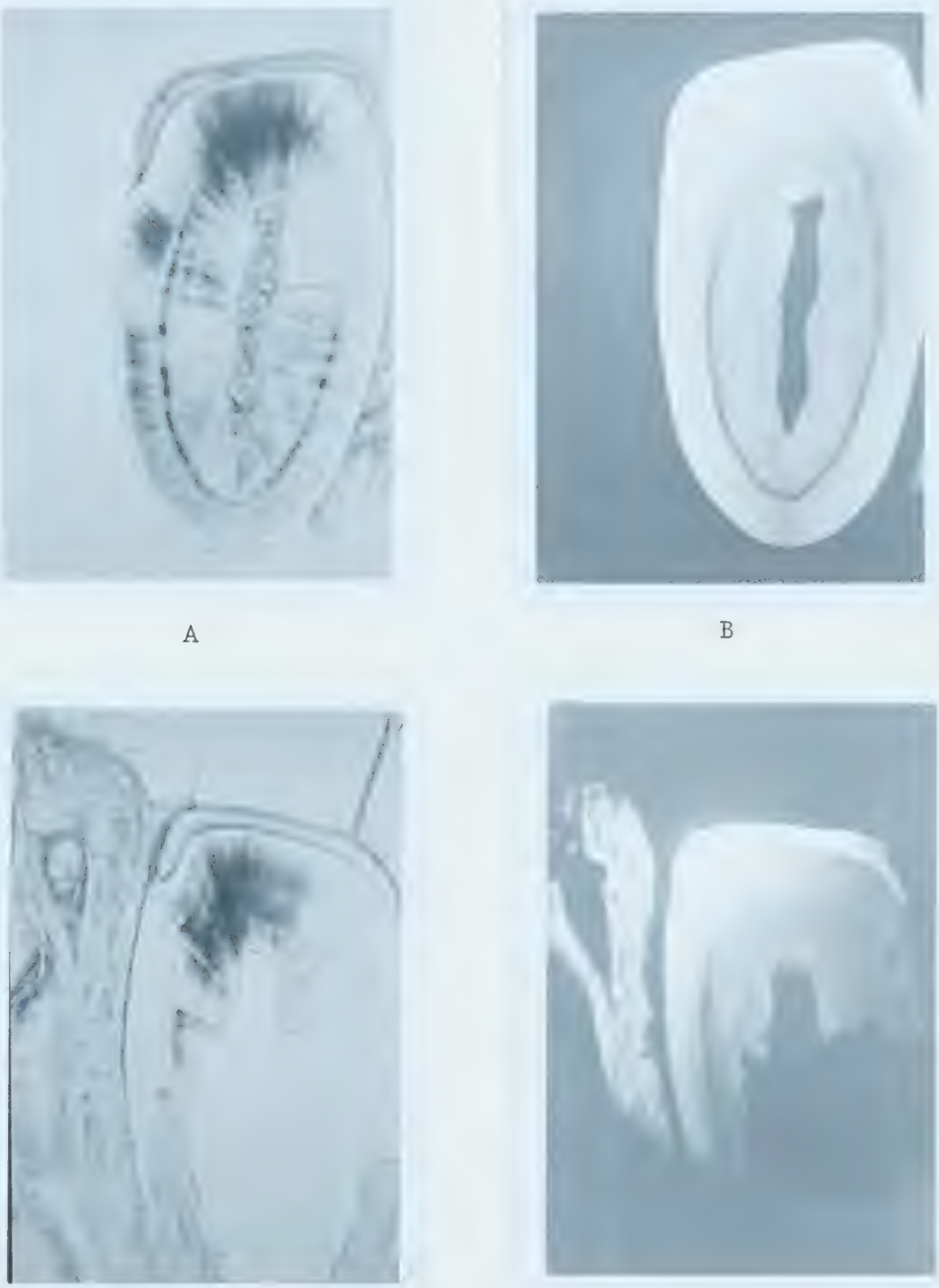
  (1) Drug used - oxytetracycline HCl (OTC)

  (2) Dentine shows light discolored brown lines

  (3) Discolored lines correspond to the fluorescent lines (Fig. 49B)

D. Microradiograph of the same section and area shown in C.

  (1) Very distinct wide radiolucent area present in the dentine, but more towards the pulpal side of the radiolucent lines.



A

B

C

D

Figure 50

Figure 51:

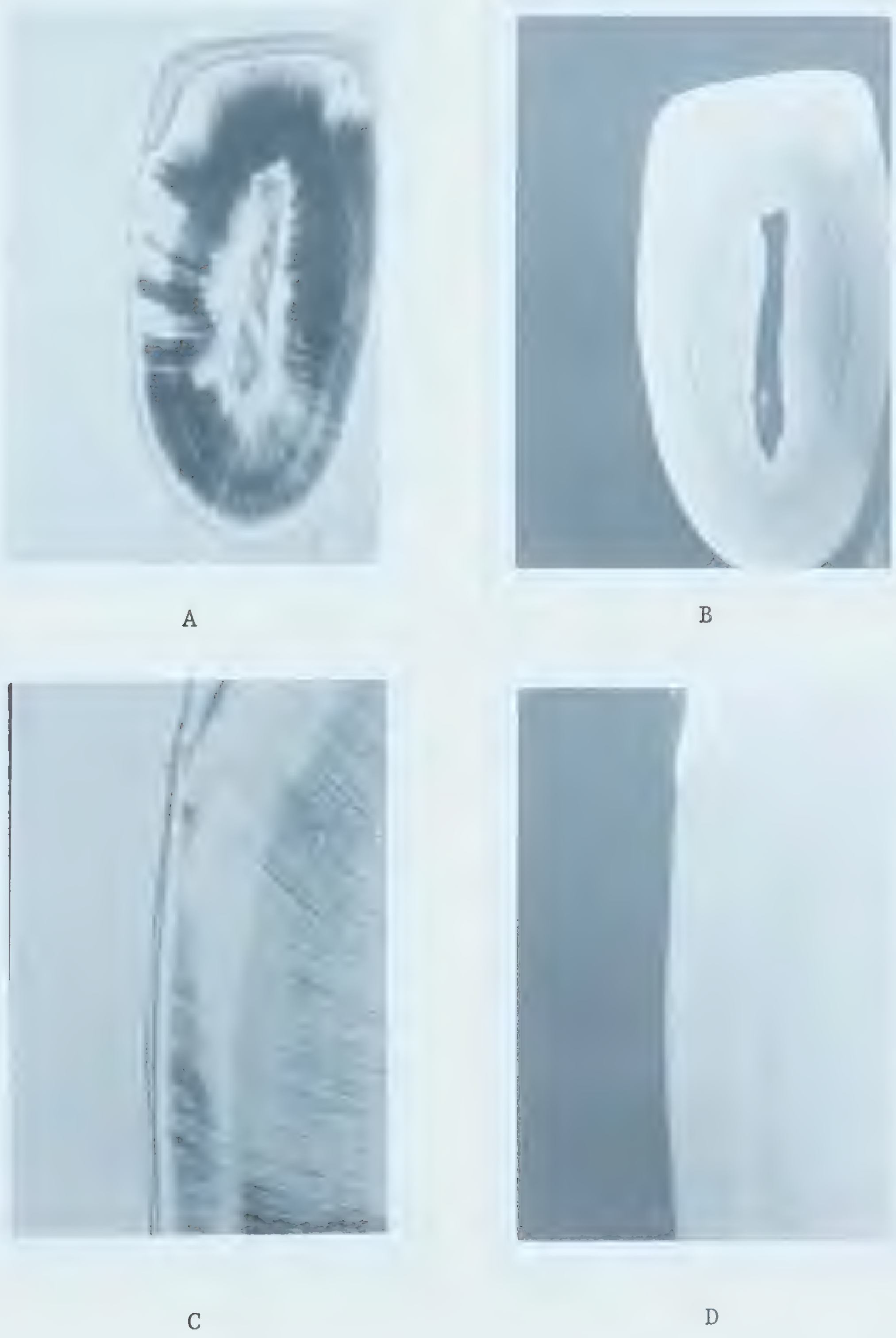
Shows photographs and microradiographs of the ground section of rat incisor teeth given tetracycline drugs at 200 mg/kg body weight dose level (equivalent dose for DCTC is 50 mg/kg). Magnification x35.

A. Photomicrograph of coronal part of the incisor.
  (1) Drug used - Chlortetracycline HCl (CTC)
  (2) Dentine shows discolored brown lines
  (3) Discolored lines correspond to the fluorescent lines (Fig. 49C)

B. Microradiograph of the same section and area shown in A.
  (1) Very distinct wide radiolucent area present in the dentine, but more towards the pulpal side of the radiolucent lines.

C. Photomicrograph of coronal part of the incisor.
  (1) Drug used - Demethylchlortetracycline HCl (DCTC)
  (2) Dentine shows clearly marked radiolucent lines
  (3) Discolored lines correspond to the fluorescent lines (Fig. 49D)

D. Microradiograph of the same section and area shown in C.
  (1) Very distinctly marked radiolucent lines present in the dentine.



A B

C D

Figure 51

DISCUSSION

A vast literature has accumulated since Duggar[1] first discovered Chlortetracycline in 1948. In subsequent years many homologues have been described. In addition to their beneficial and life saving effects in combating serious infections of pregnancy and early childhood diseases (such as cystic fibrosis of the pancreas) numerous observations of *in vivo* and *in vitro* systems have been made which establish the fact that all tetracyclines stain the calcifying tissues in the body when given during the period of growth and development and especially at the time of mineralization. Thus, all the skeletal tissues undergoing morpho-differentiation and calcification at the time when the drug is given are affected to varying degrees, depending upon the level of the doses administered. Also low doses of CTC when mixed in feed grains have repeatedly been shown to exert a stimulatory influence on the skeletal and soft tissues thus increasing the total weight of the livestock and Poultry[201-204].

Effects of the tetracyclines on growth

The findings in the present study confirm the above observations and show that low doses (10 mg/kg) cause no retardation in growth, and if anything caused some stimulatory effect on the growth of the experimental animals. These results support the observations of Jolliffe and associates[205] who found a significant increase in weight, as compared to the control group of underweight children 6 to 10 years of age, who were given 20 mg/kg of CTC daily. TC and OTC appear to have a stimulatory effect on the growth of the animals in the present study. These results are presented in Tables VI to IX and Figures 15 to 18. Based on some experimentations and mostly on observations many theories[206] have been proposed to

explain the stimulatory growth response of these drugs. One theory has suggested that the antibiotic stimulated an increased synthesis and subsequent absorption of essential nutrients in the G.I. tract of the animals. However, this does not explain the observations made by Rusoff and his associates[203], in young calves given CTC intra-muscularly, who showed that antibiotic does not alter the environment of the rumen, but felt that drug exerted its effect in the area of actual bone metabolism. The possibility of increased food consumption as a factor in weight gain has been discounted by Murray and Campbell[204] who studied the effects of CTC on the absorption of vitamin D in rats receiving rachitogenic diet. Among these conflicting reports many years later in 1963 Techernoukh and Alexandrov[207] proposed that the growth stimulating effect of the antibiotic was mainly due to a change in the intestinal flora. This change was followed by the formation of a lesser quantity of microbial toxic products and by increased assimilation of amino-acids, vitamins and growth stimulants. Furthermore, the antibiotic could also suppress the development of latent, undetected infections.

The findings in the present study that high doses cause marked reduction or total inhibition of growth at very high doses is in accord with the animal work[93,94,115,208-212] which has shown that these drugs cause marked retardation or inhibition of the growth of skeletal tissues of chick embryos. In animals, the drugs pass through the placental barrier and effect the skeletal and dental differentiating tissues to a varying degree depending upon the type of the drug and the concentration used. Our results show that there is a definite direct, though not proportional, relationship between the dose used and the retardation observed. Though the general growth trends are similar, there is a definite variation in

respect to the type of tetracycline and its effect on growth. CTC appears to have caused complete cessation of growth at 80 mg/kg and further retardation at the 200 mg/kg dose level. At the 200 mg/kg dose level, the drug was so toxic that all the animals died after three days of drug administration. Results further show that growth appears to have continued normally after the drug administration is stopped, but full recovery in comparison to control animals was not made during the time of the observations.

In order to understand how the tetracycline drugs may cause stimulation and a retardation of growth, it is essential to understand the comparative rate of uptake, distribution, accumulation, retention and excretion of the drug in various tissues. All the tetracycline drugs are absorbed from the intestinal tract irrespective of the route of administration. Parental administration of the drugs yields concentration as much as 4 to 6 times higher than achieved by the oral route. The blood concentration levels increase directly in proportion to the size of the dose at relatively low dose levels. As the dose is increased a limit is reached beyond which further increases do not lead to significantly higher blood concentrations. Tetracyclines are excreted in the bile, urine and faeces. Concentrations excreted in bile are 10 to 20 times those in the blood, and much of it is reabsorbed. The drug passes from the intestine into the blood stream, then into the liver and again back into the intestine. Twenty percent of the oral dose and fifty per cent of an I.V. dose is excreted in the urine and the difference between these two is explained by incomplete absorption from the intestinal tract or possible degradation in the body. Kunin _et_ _al_[24] studied the comparative renal clearance and plasma binding of four tetracycline drugs when given

parentally and showed that after 96 hours 18 percent of CTC was excreted in the urine in comparison to 42 to 70 percent of the other three drugs. Low renal clearance of CTC was attributed to higher degree of plasma binding. Kelly and Buyske[25] has shown that forty to fifty percent of CTC is excreted via the faeces in comparison to excretion of DCTC, 15 to 30 percent, and TC, 9 to 20 percent. Although CTC has been shown to have a high degree of plasma binding, it produces the lowest and least well sustained levels in the blood and this is probably due to its greater instability or possible degradation in the body.

Tetracyclines are localized in the hard tissues and are also found distributed in high concentrations in the reticulo-endothelial system, liver, kidney and intestinal tract. They cause G.I. tract disturbances, such as nausea, vomiting, diarrhea and also liver damage.

The exact mechanism by which these drugs could contribute to inhibition of growth, is not clear but can be theorized as follows.

(1) Inhibition or complete disappearance of the normal bacterial flora which render the bowel susceptible to invasion of other microbes.

(2) Chemical irritation due to acid pH of the drugs especially CTC which has a very low renal clearance.

(3) Inhibition of digestive enzymes (e.g. pancreatic enzymes, α amylase and lipase) thus leading to decrease in the absorption of nutrients such as glucose, starch and fats.

Thus it is possible that general inhibition in growth with all the drugs as seen in our experiments could be attributed to the 1st and 3rd factors and particular inhibition of growth with CTC in comparison to the other drugs may be due to the second factor. Low renal clearance pro-

bably lead to more G.I. irritation, excessive storage in liver, which cause liver dysfunction leading to fatty metamorphosis of the liver. This in turn disturbs the growth apparatus in some unknown way, either from a low grade calcium deficiency or by blocking metal ion co-factors necessary for action of enzymes in matrix formation. The avidity of the tetracyclines for calcium is so great that their influence on calcification has been reported by various authors[94,115,209-213].

The above discussion in respect to the effect of tetracyclines on growth so far has pointed to the fact that tetracyclines may inhibit growth and development or they may enhance it depending upon the concentration of the drug, age of the subject, environment and many other factors. At present no definite evidence is available to completely explain these findings.

Although the results of our experiments in respect to the effect on bone length do not clearly show inhibition when compared to animal growth curves it cannot be taken that the bones were not effected, but can be interpreted that whatever inhibition may have occurred during the drug administration periods might have been masked by newer growth during the subsequent rest period of 15 days.

Since the bone matrix did not appear to have been effected to any measurable extent, low inorganic contents should result from the deposition of these drugs in the bones. That this is the case has been shown by our experiments (Tables XIV to XVII) the results of which show that inorganic contents of the femora and humeri has decreased and the decreases appear to be dependent upon the doses of the drugs administered. On a comparative basis, at the 10 mg/kg dose level, CTC gave the lowest whereas at 200 mg/kg dose level TC gave the lowest inorganic contents of

the bones. These results appear to be dependent upon the renal clearance and plasma binding capacity of the drugs.

The results with DCTC shows the least effect at the therapeutically equivalent dose level of 200 mg/kg. On weight to weight basis DCTC was found to retard the growth the maximum. DCTC has a high plasma binding capacity and blood levels have been shown to remain high for as long as 48 hours after the last dose. Over a prolonged therapy, this cummulative effect may overload the excretory system of the animal and cause renal failure, which could lead to constitutional symptoms, thus effecting the growth.

Except for TC, which caused a decrease in calculated bone density both at 10 mg/kg and 200 mg/kg, the density increased in comparison to control group with all the other drugs. It is difficult to explain this result in view of our previously discussed findings that inorganic constituents of bone decrease, dependent upon dose and the type of tetracyclines used, but it can be speculated that these drugs change the biological system of the animal in some way that post drug administration period is followed by marked acceleration of skeletal growth with high density of the packing of the crystals.

Discoloration and fluorescent color intensity

The results of our experiments designed to show discoloration and fluorescence imparted the bones and teeth are presented in Tables XVIII to XXI and Figures 23 to 39. These show that there is a progressive increase in discoloration and fluorescence color intensity as the doses of the drugs were increased. As is seen from Figure 23, this increase is not strictly proportional to the doses administered. These results are in accord with that of Frost _et al_[133-137] and Bevelander _et al_[93,115].

All the drugs gave different types of fluorescent color to bones and teeth, ranging from light grey with OTC at 10 mg/kg to brownish-yellow with TC at 200 mg/kg. Stated in general terms, at each comparable dose level TC and DCTC were found to have given the maximum and OTC the minimum fluorescence in comparison to the other drugs. At any given dose level the order of discoloration and fluorescence in decreasing intensity was TC = DCTC $>$ CTC $\gg$ OTC. Our findings are in complete agreement with and further support the observations of Wallman and Hilton[74], Owen[106], Johnson and Mitchel[107], Isben *et al*[108] and Weyman[109-111] who have shown in animal and human skeletal materials that different shades of yellow which may be observed are due to different types of the tetracycline drugs. Our results further show that CTC gives a slightly brownish-yellow and not grey-brown discoloration as seen by Weyman and Porteous[109] in children. OTC was found to produce a light grey or light brown discoloration depending upon the doses administered. This finding agrees with the observations of Wallman and Hilton[81] who found that only one of eight children who had previously been given OTC had yellow teeth. Although the low number may have been due in part to the use of OTC, it is quite possible that OTC was not commonly used as CTC in the earlier years.

Over the years the observations made on children in respect to the effect of different types of tetracyclines on the deciduous and permanent teeth have shown that the teeth which were yellow in the beginning had changed to brown or grey brown on exposure to sunlight. The results of our experiments designed to show the effect of sunlight on discoloration and fluorescence are presented in Tables XX and XXI. These results confirm the observations of others[74,103,104,106,110] and show that in general all the teeth tended to turn brown. The observations made by

Isben *et al*[108] that sun decolorizes the teeth stained by CTC and OTC could not be substantiated in this study. The exposure to sunlight appears to have decreased the U.V. fluorescence only in OTC treated teeth whereas little or no change in fluorescence was observed with TC and DCTC. Our results do not support the results of Isben *et al*[108] in which CTC was found to be the least objectionable in respect to discoloration. This lack of support probably could be explained by the factors associated with the animal species, the route of drug administration and most importantly the actual doses employed in respect to body weight. Our findings show no difference in fluorescence between OTC and CTC at 10, 40 and 80 mg/kg dose levels.

The difference in behaviour among the tetracyclines in teeth may be due to a combination of several factors. The light grey or whitish yellow fluorescence as caused by OTC suggests that, quantitatively, teeth bind less OTC than the other three drugs. Also the differences noted in discoloration due to exposure to sunlight perhaps indicate the differences among the breakdown products. As Isben *et al*[108] have pointed out, the color formed after exposure to light may conceivably be related to the relative susceptibility of the anhydro derivatives to factors comparable to alkaline or acid degradation. *In vitro* experiments, however, do not show evidence for a differential type of disintegration and it is difficult to explain why TC and DCTC behave so differently toward light exposure following the administration of comparable large doses.

Our results show no gross hypoplasia or other structural defects in the incisor teeth when examined under ordinary light. There are reports [73,74,103,110] in the literature of the hypoplasia caused by tetracycline drugs but it has not been a consistent observation. The absence of hypo-

plasia when observed in gross terms does not exclude the possibility of microscopic disturbances. In fact, the finding, in the present study, that of radiolucent bands corresponding to zones of tetracycline discoloration indicates that defects in mineralization have occurred.

Findings in this study further confirm the observations made by others[106-111] that these four tetracycline drugs leave colored fluorescent markings in the forming dentine when undecalcified ground sections of the teeth were viewed under U.V. light. The color of fluorescence observed is different for each drug and each can easily be identified in the teeth. Though our results indicate that TC gives stronger or equal fluorescence in comparison to DCTC, it should be pointed out that the doses employed in this study were on the therapeutically equivalent basis. The recommended doses for DCTC are approximately one fourth the actual weights for the other three drugs. When drugs were used on weight to weight basis, DCTC was found to give the maximum fluorescence.

The intensity and type of U.V. fluorescence seen in the dentin was found to be directly related to the amount of drug administered. All the drugs left very clear fluorescent lines at 10 and 40 mg/kg dose level, with no diffuse fluorescence. At 80 mg/kg the fluorescent bands were still distinct and only TC and OTC showed a definite diffusion of the drugs in between the lines. At 200 mg/kg no distinguishable lines were observed and dentin showed a wide diffusely stained fluorescent area. TC appears to have caused some cessation in the dentine formation, as is evident by reduced band width in the dentine.

When fluorescent observations are made on whole teeth, intensity would also depend upon the thickness of the enamel and unstained dentine covering the fluorescent areas where the drug is localized. This probably

explains why deciduous teeth show comparatively more discoloration than the permanent teeth.

The above observations are similar to the one made by Bevelander[112] and Frost[133]. Bevelander found that the width of the increment of dentine and enamel which exhibit fluorescence and visible yellow color is related to the amount of drug given. For example, in their studies a dose of 10 mg/kg given to 100 Gm rat caused a fluorescent band in dentine of 5μ width but caused no visible discoloration. 100 mg/kg to a 100 Gm rat resulted in fluorescent band 25μ width. Frost[133] observed that band width is a direct function of duration of doses and rate of mineralization of tissues, whereas intensity of fluorescence is a function of dosage per day. Thus it appears that fluorescent intensity, banding and diffusion in the adjacent area may be directly related to the concentration and duration of the tetracycline in the blood. Studies of Buyske _et al_[25], Kelly _et al_[26] and Eisner and Wulf[27] have indicated that the binding of tetracyclines in bone was in decreasing order, DCTC, CTC and TC. (No data are available for OTC.) If fixation of the drugs in the skeletal tissue is by chelation, as has been described by Regna _et al_[128], Albert[129], Ishidate and Sakaguchi[214], Kelly and Buyske[215], then the amount fixed in the calcified tissues will be related to the chelating ability of the particular tetracycline, the availability of suitable binding sites in the particular stage of development of the tissue and the concentration of the drug in the fluids around the bones and teeth. The presence of well defined fluorescent bands in dentine can be explained on the basis of observations of Buyske _et al_[25] that this chelation phenomenon is an immediate consequence of the presence of tetracycline in the blood stream. The diffusion observed in dentine between the fluores-

cent lines and after the drug was withdrawn can possibly be due to either the persistence of tetracyclines in the blood stream for much longer time or leaching out of the initially deposited tetracycline into the newly mineralized or mineralizing tissues. The last effect may well occur in the bone which has much more profused blood supply, and lower density of the apatite crystals. This view is consistent with work of Buyske *et al*[25] who has shown that bone has a capacity to bind tightly only a portion of the amount of tetracycline initially absorbed. The rest is loosely held and is removed rapidly by diffusion into the blood. The possibility this may also occur in teeth, is unlikely, because except with TC no diffuse staining was observed in the previously mineralized though not completely matured dentine when the first dose was administered.

It appears more likely that diffused staining is due to persistence of tetracyclines in the blood. Buyske *et al*[25] have shown that there is a direct relationship between blood concentration of the drug and the bone deposition of the drug. They injected intraparitoneally TC varying from 10 mg/kg to 150 mg/kg in rats and measured serum concentration of the drug, as well as drug deposition in the bone at 2, 4, 6, and 24 hours. The results showed that at 10 to 30 mg/kg dose level the maximum serum concentration is reached in two hours and falls very rapidly after six hours and is virtually eliminated at 24 hours. As the doses are increased to 150 mg/kg the serum level concentrations are highest at 4 hours (54 μg/ml) and 24 hours later 66 percent of the concentration initially obtained was still present, i.e. a level of 35 μg/ml which is comparable to the 38 μg/ml caused by the 100 mg/kg dose after 4 hours. Thus a cumulative deposition of the drug in part may explain the diffuse appearance

observed in calcified sections of teeth following high doses. Furthermore the smaller width of fluorescent bands in dentine observed with TC, OTC and DCTC at very high dose levels might indicate interference with the matrix formation in some unknown way.

Though no fluorescence was seen in the enamel at 10 and 40 mg/kg dose level, faint whitish yellow fluorescence was observed at the 200 mg/kg dose level with all the four tetracycline drugs. These observations provide support to the works of Storey[96], Harcourt[97], Weyman[98] and Owen[106] who also showed the occurrence of fluorescence in enamel of dogs and human teeth.

SUMMARY

Seventy two Sprague Dawley rats were used for this study. Four tetracyclines namely Tetracycline HCl(TC), Oxytetracycline HCl(OTC), Chlortetracycline HCl(CTC) and Demethylchlortetracycline HCl(DCTC) at 10, 40, 80 and 200 mg/kg dose levels were investigated with respect to their effect on animal and bone growth, and to the color and intensity of fluorescence produced by each.

The results show that all the drugs caused inhibition in growth at 80 mg/kg and 200 mg/kg dose levels, but CTC was observed to be the most toxic. At normal dose levels (40 mg/kg) all the drugs appear to be relatively safe and without any deleterious effect on growth. The administration of low doses (10 mg/kg) seems to have some stimulatory effect on the general growth of the animals.

Although there was no appreciable change in the length measurements of long bones, a definite decrease in inorganic constituents of bones was observed at all four dose levels tested. At high dose levels (200 mg/kg) the maximum decrease of 13.35 percent was noted following administration of TC. OTC resulted in decrease of 8.45 percent whereas DCTC caused decreases of approximately 5 percent.

At 200 mg/kg dose level, administration of TC resulted in a definite decrease in weight, volume, density and inorganic constituents of bones and this effect appears to be less marked with the other three drugs. DCTC affects bone the least in this respect.

Gross examination of long bones and teeth showed no visible anatomical defects or abnormalities. Under ordinary light the changes ranging from light grey (OTC, CTC at 10 mg/kg) to light brown (OTC at 40 mg/kg) to deep brown yellow (TC, DCTC at 200 mg/kg) were seen. At any

given dose level, the order of discoloration was $\mathrm{TC} = \mathrm{DCTC} > \mathrm{CTC} \gg \mathrm{OTC}$.

Under U.V. light, bones and teeth showed brilliant yellow fluorescence in all the drug groups except OTC. The degree of observed fluorescence was directly dependent upon the doses of the drug administered. At the 10 mg/kg dose level the fluorescence was whitish blue, which changed to deep orange at 200 mg/kg dose level.

Histological examination of ground sections of teeth showed clear and distinct fluorescent color markings reflecting daily dosage pattern at the 10 mg/kg and 40 mg/kg dose levels and as the dosages were increased to 200 mg/kg, the markings became diffuse and indistinct and the total area occupied by these markings in the dentin appeared to have become wider relative to the area observed at the 10 mg/kg and 40 mg/kg dose levels. The color of the fluorescence varied from light yellow at low dosage to deep orange at high dose levels. At any given dose level the intensity of fluorescence was $\mathrm{TC} = \mathrm{DCTC} > \mathrm{CTC} \gg \mathrm{OTC}$.

At the therapeutically equivalent dose level the TC was found to give the maximum fluorescence, whereas DCTC gave the strongest and most intense fluorescence when drugs were used on an equivalent weight basis.

Exposure to sunlight appeared to have increased the discoloration and observed fluorescence slightly in all the drug groups except OTC group where a little or no change was observed.

No fluorescence was observed in the enamel at 10 mg/kg and 40 mg/kg dose levels. All the four tetracycline drugs exhibited a definite whitish yellow fluorescence in the enamel at 200 mg/kg dose level.

At high dose levels (200 mg/kg) only TC appeared to have reduced the width of the dentin formed during the time the drug was administered.

Micro-radiographic examinations of the ground sections of the teeth

shows faint but perceptible radiolucent lines in the dentine corresponding to discolored and fluorescent lines at 10 mg/kg, 40 mg/kg and 80 mg/kg dose levels. At high dose levels (200 mg/kg) the perceptible lines seem to have merged into a wide and very distinct radiolucent area.

A discussion of the findings is given which draws the conclusion that at normal dose levels all the four drugs are relatively safe with no adverse effect on growth. Furthermore the degree of observed discoloration and fluorescence in teeth is directly dependent upon the doses administered.

OTC gives the minimum and less objectionable fluorescence, thus making it the drug of choice in clinical practice as far as discoloration is concerned.

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